Aus dem Institut für Veterinärpathologie
der Vetsuisse-Fakultät
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Direktor: Prof. Dr. A. Pospischil

Arbeit unter Leitung von Prof. Dr. F. Ehrensperger

Effect of antiviral substances on Borna Disease Virus *in vitro*

INAUGURAL-DISSERTATION

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vorgelegt von

Francesca Del Chicca

Tierärztin aus Parma, Italien

Genehmigt auf Antrag von

Prof. Dr. F. Ehrensperger, Referent

Prof. Dr. A. Pellegrini, Korreferent

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1. Abstract

Borna Disease is a severe, immunopathological disorder of the central nervous system, caused by infection with Borna Disease Virus. The main known naturally affected animal species are horses and sheep in endemic areas in central Europe. The detection of BDV in these hosts is achieved by histological, immunohistochemical and serological approaches and/or PCR-based technologies.

In this study, the effect of some proteins and drugs *in vitro* on two BDV infected cell lines (MDCK and Vero cells) was tested. The method is based on an enumeration of the infected cells, labelled with immunofluorescence compounds.

A tracking method using a fluorescent cell marker (CMTPX) was developed, in order to find out if the reduction of the number of infected cells was due to a cytostatic effect or to an impairment of the virus spread from cell to cell.

This cell tracker enables, by a double staining technique, to appreciate at the same time the BDV infected cells and the cell lineage. The results obtained show that Ribavirin and Cytosine β-D-arabinofuranoside have an inhibitory effect on BDV in culture, whereas Amantadine had no consistent effect on BDV replication *in vitro*.

The effect of Ribavirin was mainly directed to inhibit the virus spread through the cell contact, while Cytosine β-D-arabinofuranoside inhibited at the same time the replication of infected cells as well as the virus passage from cell to cell.

None of the proteins tested, having antiviral effects against other viruses, presented antiviral activity against BDV.

2. Introduction on Borna Disease Virus (BDV)

2.1 History

Borna Disease (BD) is a unique neurological disease caused by infection with an RNA virus, Borna Disease Virus (BDV). The oldest report describing the typical characteristics of BD are found in 1660, speaking of pain which pushes the horses or makes them dull and dumb [47].

A veterinary handbook published in 1785 [2, 85] contained an accurate report of the clinical syndrome, implicating unsatisfied sexual desires or overfeeding in its pathogenesis and suggested colourful therapies such as phlebotomy, plucking air from selected locations, and threading a rope coated Spanish fly cream through the subcutaneous tissue of the infected horses [79].

The disease was first described in details approximately 200 years ago in horses in southeastern Germany as a syndrome of agitated aggressive behaviour that progressed over a period of weeks to inanition and death [2, 85].

The disease became more important in 1885, when a large number of horses died in the town of Borna (Saxony, Germany), during an epidemic outburst of BDV-induced disease [115, 129]. After this time point, the name of Borna Disease was adopted and in 1896 for the first time, the definition of Borna Disease appeared on the official veterinary review in Berlin (Berliner Thierärztliche Wochenzeitschrift) referred to a cerebrospinal meningitis in horses [76].

In 1909, Joest and Degen discovered intranuclear inclusion bodies associated with the disease in the ganglion cells of the Hippocampus, and for the first time a *post mortem* diagnosis of the BD was possible [71].

The nature of the causing virus had remained obscure until the etiology of BD was established in 1925, when Zwick and Seifried proved its transmissibility [148].

In recent years, the number of animals diagnosed with classical BD in Europe was relatively low, usually affecting fewer than a total of 100 horses and 100 sheep every years [26, 39, 63, 129] .

To our knowledge, until recently, no confirmed cases of BD had been reported in horses or sheep outside the endemic areas, that other than certain areas of Germany [39], also includes part of the upper Rhine valley between Switzerland, Austria and the Principality of Liechtenstein [24, 129, 143] .

However, this apparent geographic restriction may reflect lack of reliable methods and reagents for diagnosis of the infection or insufficient disease awareness.

Recently, the interest in BDV has increased because it has been recognized that that geographic distribution of the natural infection and the host range spectrum are much larger than previously appreciated [79], including sheep, cattle, cats and even human in other parts of Europe as well as in North America and Asia [85].

2.2 Aetiology

Borna Disease Virus represents the only member of the family Bornaviridae that is part of the order Mononegavirales, which includes the families of Filoviridae, Paramyxoviridae and Rhabdoviridae, BDV is an enveloped, non-segmented, single- and negative-stranded RNA virus (NNS RNA) [146]. There are four known BDV-strains, called Borna V, Borna HE/80, Borna No/98, Borna H1766; the genomes of these strains have been sequenced and showed a very high sequence conservation, which is uncommon for RNA virus [22, 29, 65, 100, 108].

A characteristic of the BDV is that its replication and transcription of the genome occur in the cell nucleus: the only other negative-strand RNA virus know to transcribe in the cell nucleus is influenza virus [85]. The genome size is about 8.9 kilo bases, and the organization of the six major ORFs is similar to that of the others Mononegaviruses [123].

Electron microscopic studies of negative-stained cell-free BDV infectious particles have shown a spherical morphology with a diameter ranging from 70 to 130 nm [50, 77, 146]. These particles contain an internal electron-dense core (50 to 60 nm) and a limiting outer membrane envelope, which appeared to be covered with spikes approximately 7.0 nm long [77, 121].

Virus infectivity is rapidly lost at temperatures higher than 56°C, as well as at pH's below 5 and above 12, and by treatment with organic solvents, detergents, formaldehyde and exposure to ultra violet radiation [33, 121].

2.3 Epidemiology

2.3.1 BDV in animals

BDV infections mainly affect horses and sheep but the disease involves also other animals, although the incidence appears to be very low. BDV was found in donkeys [24, 147], goats [24] and cattle [17, 25] with neurological disease and prominent mononuclear infiltration of the CNS. It has also been suggested as agent of the 'staggering disease' in cats [87], but its effective involvement in these cases is still matter of discussion. BDV was also identified in lynx [12], rabbits [92], ostriches [5, 89], tree-shrews [128] and in many laboratory animals, such as rats, mice, rabbits and gerbils [79, 121].

Specific BDV-antibodies were detected in horses' sera from Holland, Luxemburg, Poland, Russia, Israel [61], Japan [95] and USA [73], but in none of these cases clinical signs of the disease were present. These reports of asymptomatic natural infection of animals suggest that the virus may be even more widespread than previously thought [125].

It has been assumed that a possible way of infection can be the transmission of BDV through body secretions (mainly urine and faeces, but also salival, nasal or conjunctival secretions). In fact, several studies detected BDV RNA in body secretions. [8, 63, 114, 116]

A direct contact with these secretions or exposure to contaminated food or water is regarded a successful way of transmission [114, 120].

The natural source of infection has still not been determined [129]. Anyway, rodents are considered potential reservoir and vector, because it has been demonstrated that the urine of experimentally infected rats contain high BDV titers, and inoculation of adult black hooded rats with BDV induce persistent infection [59]. Nevertheless, their rule in the Borna epidemiology has not yet been demonstrated.

Very recently, shrews have been recognized as vectors [64].

Other wild species reported that could be implicated in the BDV epidemiological cycle are wild birds [13, 31] and ticks [121].

2.3.2 BDV and zoonotic potential

It has been suggested that BDV infection might contribute somehow to the syndrome of major depressive illness by altering neuronal cells in the limbic system [18]. Behavioural abnormalities and disturbance have been observed in infected rats. The behaviour disturbances were considered to be reminiscent of affective disorders such as bipolar and monopolar. This observations led to the question whether human patients with similar disorders might be infected with BDV [85]. Representative longitudinal studies showed that episodes of depressive illness in humans as well as apathetic phases in infected horses were accompanied by BDV-protein expression and followed a similar clinical course [19]. Seroepidemiologic studies have demonstrated BDV-specific antibodies in sera of psychiatric and neurological patients in Germany, Japan and the United States [21, 44]. Others studies [119] showed a significantly higher prevalence of BDV serum antibodies among hospitalized psychiatric patients and a moderately higher seroprevalence among neurologic patients than among controls. Investigations of CSF's of BDV-seropositive patients acutely ill with psychiatric disorders (mainly schizophrenia and affective psychosis) showed intrathecally synthesized anti BDV- specific immunoglobulin (IgG) in 25% of the patients [115]. Also the chronic fatigue syndrome has been related with a possible BDV infection [96].

A slightly higher level of seroprevalence was found among psychiatric patients in an area with high incidence of natural BDV in horses (South of Germany) [115].

In the last years, evidence of human infection of BDV has been sufficiently confirmed, but a causal link with neurological diseases remains difficult to prove [65]. In fact, the etiopathogenic relationship between virus and human disease remains a major question [20, 85, 140].

2.4 Clinical manifestation in animals

Clinical symptoms exhibited by horses and sheep with BDV are quite variable and can vary from behavioural anomalies to severe neurological disorder and death, paralleling to the severity of the inflammatory reaction in the CNS. They are species, age and immune status dependent [23, 118] .

The incubation period is also noticeably different. It can range from 2 weeks to few months and BDV infections in horses can also be clinically inappearent [121].

Spontaneous BD in horses and sheep typically begins with a short prodromal stage of depression and anorexia. This is followed by overt disease, the hallmarks of which are somnolence, ataxia, dysphagia and multiple neuronal degeneration. Other non specific symptoms are hyperthermia, colic and constipation, or typical signs of encephalitis (with movement and posture disturbances, proprioceptive deficits, lethargy, hyperexcitability). The course of the disease is progressive over 1 to 3 weeks and usually leads to death [23, 40, 53, 113].

The reported mortality rate is about 80-100% in horses and about 50% in sheep [115].

In experimental infections of laboratory animals all the clinical manifestations and the incubation period appear to depend on the animal species, viral strain and host immune status [31].

2.5 Immunopathogenesis

BD is defined as a non purulent polioencephalomyelitis caused by a T cell-dependent immune mechanism [32].

BDV is a highly neurotropic agent. It gains access to the CNS after a receptor-mediated endocytosis [49], probably by intraaxonal migration through the olfactory nerve, the olfactory neuroepithelium or nerve endings in the oropharyngeal and intestinal regions [118]. Virus spreads throughout the CNS by intraaxonal transport and centrifugally into the peripherals nerves. Antibody titres in naturally infected animals are relatively low and are found in sera and cerebrospinal fluid. Whether BDV-specific antibodies are neutralizing is not certain [86, 97].

In immunocompetent laboratory animals, as shown in extensive studies on the Lewis rat, no infectivity was found in extraneural tissue at any stage of infection, while in newborn animals the virus spread is throughout the whole organism [97].

Despite productive virus replication in the CNS, immunocompromised and newborn animals do not become ill with BD or encephalitis, suggesting the hypothesis of a virus-induced cell-mediated immunopathological basis of BD. In fact, T cells play an important role in the immunopathological reaction and in the set on of the disease [115].

In experimentally infected rats, the cellular mediated immune reaction has been characterized by perivascular infiltration of CD4 positive and CD8 positive T cells (CD4 positive cells are prominent), with presence of natural killer cells and macrophages [132].

The same pattern of lymphocyte phenotypes has been observed in horses and sheep. In brief, the majority of inflammatory cells in perivascular infiltrates as well as in parenchymal and meningeal infiltrates were CD3 positive. CD4 positive cells outnumbered CD8 positive cells in perivascular infiltrates as well as in the parenchyma. Macrophages were seen less often and B-cells or plasma cells were demonstrated at lower numbers [23].

Other studies confirmed that BD in rats appears to be a CD4 positive T-cell dependent immunopathological disease, in which CD8 positive T-cells and /or CD8 positive T-cell- mediated cytodestructive mechanism lead to tissue damage [91], brain atrophy and clinically to organ dysfunction and manifested disease [121, 130].

2.5 Diagnosis

A definitive clinical or *intra vitam* diagnosis of BD is not possible yet, even if meningoencephalitis induces, in the acute phase, a slight alteration of protein concentrations in cerebrospinal fluid. These indicators are not specific for BD but are indicators of viral meningoencephalitis [15, 121].

Serological diagnosis can be applied to living animals by antibody detections in blood and/or CFS. Western blot [63], ELISA [40] and immunofluorescence assay (IFA) [62] can be used to confirm a diagnosis and the latter method is commonly considered the most reliable [31]. However, it has been proved that antibodies could not be regularly found in animals where the diagnosis of BD was histologically confirmed [24].

On the histopathological level, various degrees of encephalitis can be observed. Typically, BD is characterised by disseminated mononuclear meningitis and poliencephalomyelitis with subsequent neuronal degeneration. Usually, a site predilection for areas of the limbic system, particularly the hippocampal formation can be noted, whereas the brain stem and cerebellum are relatively spared [23].

Joest-Degen inclusions bodies in nuclei of infected neurons have been used as BDV-specific markers, but they are not always observed [31, 52].

Viral isolation from brain tissue is a non reliable method, due to the low number of infectious particles *in vivo* [58, 129]. *In vitro*, BDV can be easily cultivated on Vero cells (monkey kidney cells) and MDCK (dog kidney cells). BDV persistently infected cells do not show any cytopathic effect [31].

RT-PCR or RT-nested-PCR [83] can be used for demonstrations of viral RNA from brain or blood sample. This is a very sensitive technique, but prone to cross-contamination between sample as well as laboratory contamination. Moreover, it is not possible to detect variant strains that have altered sequences in the target gene [31].

2.7 Antiviral substances and treatment

To date little is known about antiviral substances that may inhibit the replication of BDV, thus there is no effective treatment of BD. Different compounds have been investigated against BDV. It has been shown that high concentration of Ribavirin strongly inhibited replication of BDV in persistently infected MDCK cells [93] and the nucleoside analog 1-β-D-arabinofuranosylcytosine (or Cytosine β-D-arabinofuranoside, CAF) efficiently inhibits BDV replication [7]. Furthermore, Amantadine sulfate has been reported to inhibit BDV replication in persistently infected human cells in culture as well as in a psychiatric patient [16]. However, this inhibitory effect of Amantadine toward BDV could not be confirmed by other studies [28, 55, 131].

A mannose derivative, 1B6TM has been proved to prevent *de novo* infection with BDV of cultured human oligodendroglial cells [134]. Interferon antiviral activity against BDV has been demonstrated in some cell lines [54]. The immuno-supressive drug cyclosporine A was also able to prevent BD in experimentally infected laboratory animals [133].

2.7.1 α-D-Mannose

α-D-Mannose occupies the terminal position on the N-linked carbohydrate side chain of BDV-specific gp17 [135]. A hydrophobic derivative of this sugar residue, the 1-0-benzyl-6-0-trityl-α-D-mannopyranoside (1B6TM), showed a potent and a selective inhibition of BDV replication *in vitro* without any cytotoxic effect. Because BDV particles contain terminal α-D-mannose residues the antiviral effect of 1B6TM is likely to be highly specific to those viruses in which such terminal carbohydrates are essential for the infection process [134].

2.7.2 Interferon

Interferon-α/β (INF) is a natural antiviral agent, synthetized in response to most viral infections by a large number of vertebrate cells [34]. A previous work showed that exposure of primary rabbit brain cells to exogenous INF prevented their infection with BDV, while IFN treatment caused no inhibitory effect on BDV persistently infected rat lung cells [142]. It remained unclear, however, whether the poor performance of INF in persistently infected cells indicated that early steps of the BDV replication cycle were the exclusive targets of this antiviral cytokine. A later study has shown that IFN can strongly reduce the virus load in persistently infected monkey Vero cells but not in rat C6 cells. That indicates that steps of the BDV replication cycle of virus entry are affected in IFN- responsive cell lines [54]. Unlike all other cells line were tested, C6 cells could also not be protected with IFN from *de novo* infection with BDV, indicating that it lacks at least one component of the IFN system that inhibits BDV. From these last results, it cannot be excluded that IFN plays a role during the spread of the virus to the CNS after natural infections with BDV via peripheral nerves [54].

2.7.3 Cyclosporine A

As already mentioned, in rats persistently infected with BDV, severe neurologic disorders and occasional death are the consequence of a T cell-mediated immunopathologic reaction in the brain. It has been proved that the pathologic alterations in the brain and, as a result, BD can be prevented by treatment with the immunosuppressive drug cyclosporine A (CSA) [133]. CSA is an immunosuppressive agent that has been proved very useful also in the treatment of experimental disease caused by immune mechanism [122]. In experimental models, the onset of the disease has been clearly shown to coincide with histologic alteration 15 to 28 days after intracerebral infection of rats. After immunosuppression with CSA, infected rats developed a persistent infection yielding comparable titers of infectious virus in neural tissue as compared to nontreated BDV infected animals. However, such immunoincompetent rats show no clinical symptoms or lymphocytic infiltrations despite the presence of virus in the brain [60].

2.7.4 Ribavirin, CAF and Amantadine

The antiviral effectiveness and the action mechanism of Ribavirin, CAF and Amantadine are discussed later in details (see 2.9.1-2.9.3).

2.7.5 Liquor filtration

An alternative adjuvant therapy that has been tested in the last years is the liquor filtration [10, 11]. It has been described as an experimental method in human patients affected from therapy-resistant schizophrenia, psychoses and in diseases where an immunopathogenetic genesis was recognised, like in BD [9].

From previous studies, an earlier unknown therapeutic effect of a cerebrospinal fluid filtration (CSFF) in therapy-resistant major depression has been suggested for different reasons: firstly, therapy-resistant major depression and comorbid symptoms improved with CSFF; secondly, test performance improved with CSFF; lastly, relapse occurred after discontinuation of medication but therapeutic effects were repeated with a second CSFF serie [9].

The rationale for a CSFF was in short: therapy resistance, symptom characteristics, BDV serum and/or cerebrospinal fluid antibodies, known immune pathogenesis of BD. The first schizophrenic patient submitted to this procedure, improved with CSFF and was clinically stable, since 2.5 years free of medication, fully working and socially integrated, himself feeling healthy [9].

However, the exact mode of action of CFSS remains elusive. Filtering out toxic or blocking factors may explain a rapid short-lived but not lasting improvement. It has been speculated whether removing one endocaine may help to normalize immunological function within the central nervous system [9].

2.8 Antiviral Proteins

Since the 1950s, drugs capable of inhibiting virus replication were described in the scientific literature, but most of these antiviral chemotherapeutic agents are characterized by limited efficacy, adverse side effects and suboptimal pharmacokinetics [103]. Recent technology advances have facilitated greater understanding of the molecular biology and biochemistry of the viral enzymes involved in the viral life cycle. In particular, viral enzymes which are essential for the production of infectious virus represent potential therapeutic targets.

During the last decade, preclinical research efforts have centred on virus encoded proteases as potential targets for antiviral intervention [36, 74, 80, 103].

Proteins or modified proteins have also been reported to possess antiviral activity [102]. In our investigations, we decided to test against BDV proteins that were shown to have in previous studies a marked antiviral activity against several viruses like Human Herpes Simplex Virus type 1 [101], HIV and Human Cytomegalovirus [56] and Adenovirus [4].

2.8.1 Bovine lactoferrin

Bovine lactoferrin has been recognised as a potent inhibitor towards different enveloped viruses such as Herpes Simplex Virus (HSV) 1 and 2 [57], Human Cytomegalovirus [56], Human Immunodeficiency Virus [112, 139], Human Hepatitis C Virus [67, 145], Respiratory Syncytial Virus [110] and Hantavirus [94]. Moreover, its antiviral activity against two naked viruses, SA-rotavirus and Poliovirus type 1, has been also demonstrated [90, 136]. For all the investigated viruses, lactoferrin exerted its antiviral activity in the early phases of infection. Since lactoferrin is known to bind cell surface gycosaminoglycans and low-density lipoprotein receptors, which acts as binding sites for HSV 1 and HIV [117, 124], its inhibiting activity on these viruses has been ascribed to a competition for cell receptors. However, even thought a direct interaction between lactoferrin and viral particles has not been ruled out till now. For Rotavirus and Poliovirus, which interact with carbohydrate moieties other than glycosaminoglycans, it has been suggested that lactoferrin could bind to viral particles, in a similar manner as it has been reported for some enveloped viruses

[139, 145]. Interestingly, a further effect on a later intracellular step of virus infection has been also described in Rotavirus infection [137].

2.8.2 β-lactoglobulin

β-lactoglobulin has shown to possess inhibitory effect *in vitro* against several viruses like Herpesvirus [99] and HIV [14, 98].
Several studies have shown that acylated plasma and milk proteins with increased negative charge have a potent antiviral activity, in particular against HIV-virus [138]. The antiviral effect seems to be positively correlated with the number of negative charges introduced into the various polypeptides: in fact, proteins with a high content of basic amino acids in which all of the available epsilonNH_2 groups were anionized yielded the strongest antiviral activity [138]. The mechanism of action of acylated proteins and β-lactoglobulin consists to block the fusion process between the cell membrane and the virus [138].

2.8.3 Bovine serum albumin

Bovine serum albumin has been demonstrated to improve the cytoplasmatic delivery of a phosphodiester oligonucleotide (PO) positively influencing at the same time the antiviral activity *in vitro* against Human Cytomegalovirus [3]. Human serum albumin has been investigated as well [69]. Its modification by introduction of a single or two carboxylic groups (Suc-HSA and Aco-HSA) yielded strongly negative charged compounds. In studies against HIV-1 virus, it has been demonstrated that the mechanism of action is an inhibition of a post-binding virus-cell fusion, probably due to interference with the gp41-mediated fusion process. Moreover, Aco-HSA was also able to inhibit virus-cell binding by shielding viral gp120 [69].

2.8.4 Lysozyme

Lysozyme is widely known to have both bactericidal and antiviral activity. It has been proved to inhibit DNA and RNA synthesis, to damage the outer membrane of E. coli [106]. The block of macromolecular synthesis and permeabilization of the inner membrane impair the stability of the microorganism and lead to bacterial death. Lysozyme exerts a membrane disturbing activity on fungal and plant cells as well [38]. Moreover, it inhibits the formation of syncytia in cell monolayer infected with Herpes Simplex Virus [27].
On the other hand, lysozyme has already been found strongly toxic for the Vero cells [102], toxicity has been confirmed in this study on MDCK cells.

2.8.5 α-lactalbumin

Also α-lactalbumin (together with β-lactoglobulin) both native and after modification with 3-HP (see 2.8.6), have been tested against HIV-1 [14]. The introduction of multiple negatively charged carboxyl groups along the polypeptide backbone leads to repulsion within the protein molecule and this is likely to affect the specific tertiary, and perhaps also secondary structure of the protein.
One of the most potent inhibition of HIV-1 replication was obtained from 3-HP α-lactalbumin. This compound showed a low cytotoxicity [14].

2.8.6 Chemical modification of the proteins

3-Hydroxypthalic anhydride (3-HP) is an aromatic anhydride that reacts with the terminal NH_3-group of lysin. Through this reaction, hydrophobic and negatively charged groups are introduced in the lysine molecule and consequently in proteins [3, 14, 78, 101, 138]. Chemical modification of β-lactoglobulin through reaction with 3-HP yield a compound strongly active against HIV [98], Herpes Simplex Virus-1 and –2 [99] and *Chlamydia trachomatis* [70].
More in general, some 3-HP-modified proteins strongly inhibit the multiplication of Herpes Simplex Virus-1 and this confirmed that the introduction of hydrophobic

and negatively charged residues in the polypeptide chain could be a useful procedure to confer antiviral activity to a protein [101].

Negative charge and hydrophobic interaction between antiviral compounds and virus envelope proteins have been suggested as a possible active principle for this antiviral activity [99].

However, the fact that 3-HP-modified proteins failed to inhibit, beside BDV also Bovine Parainfluenza Virus type 3 and Porcine Respiratory Coronavirus, all enveloped viruses as well, indicates that an non-specific damage of the virus envelope, caused by hydrophobic and electronegative interaction between the 3-HP-proteins and the envelope proteins, is unlikely [102].

Previous studies confirmed that β-lactoglobulin and α-lactalbumin after modification with 3-Hydroxypthalic anhydride (3HP) [14, 78], human serum albumin, modified in strongly negative charged compounds [69] and lysozyme or modified lysozyme fragments [102] possess antiviral properties.

2.9 Chemical substances

2.9.1 Ribavirin

Ribavirin is a purine nucleotide analog that bears a close structural resemblance to guanosine. It has been often reported as an antiviral active substance against different viruses, like Respiratory Syncytial Virus, Hepatitis C Virus [75, 105] and BDV [72].

Previous report demonstrated that Ribavirin possess antiviral activity against two different strains of BDV (strain V and He/80) present in two neural cell lines of different lineage (human oligodendrocytes and rat glia) [72].

Ribavirin is transported into cells and converted by cellular enzymes to mono-phosphate derivates. It has been proved to be active against both RNA and DNA virus replication [104] and it shows a double mechanism of this inhibitory effect: firstly an inhibition of polymerase [93, 127]; secondly, an inhibition of transcription and capping of viral mRNA, due to a reduction of the intracellular GTP pool [42, 72]. More in detail, the target reaction is the mRNA capping guanylation after phosporylation at the 5' position [41, 51, 144].

There is evidence that both mechanisms are operative in BDV-infected cells treated with Ribavirin. The treated cells had lower levels of viral mRNAs, a result consistent with inhibition of transcription. Moreover, an even more striking effect was observed with respect to viral titers. This latter finding is in accord to an additional functional deficit in BDV transcripts due, as mentioned, to inhibition of capping and reduced efficiency of translation. Support for the hypothesis that Ribavirin acts through interference with GTP/dGTP biosyntesis has been found in other viral systems. Measles virus is an example where the antiviral effect of Ribavirin *in vitro* is abrogated by addition of guanosine but not adenosine [144].

2.9.2 Cytosine β-D-arabinofuranoside (CAF)

CAF is a well known specific inhibitor of viral and cellular DNA polymerase [46]. It is a nucleoside analogue that differs from cytosine by the presence of a hydroxyl group at the 2' position of the sugar residue. Its active metabolite, Ara-CTP, inhibits cellular and viral DNA polymerases [46]. Nevertheless, previous studies proved that it could also inhibit BDV, a negative-stranded RNA virus that synthesizes only RNA [7].

The inhibitory effect of CAF on BDV replication and dissemination might be caused by direct effects on the viral replication machinery or by interference with host cell functions that the virus needs for its replication or spread. Even if CAF is known to have an antimitotic activity, that is the basis for its use in the treatment of leukemias. It has also been recently assessed that the antiviral effect of CAF against BDV cannot be attributed to its effect on the host cells at the concentration tested, because the BDV spread has resulted to be independent from the presence of antimitotic drugs [7]. So, it has been suggested that its effect is exerted directly on the viral replication machinery. In fact, CAF could directly inhibit the activity of the L- polymerase or alternatively it could exert a mutagenic effect during RNA synthesis mediated by BDV-L polymerase, resulting in the generation of high levels of non-functional viral genomes [7].

Others authors recently reported CAF to have a strong activity also against BDV probably due to an inhibition of a RNA-dependent RNA polymerase as well [35, 109, 141].

More in detail, CAF has been reported to exert not only a progressive inhibition of both genomic RNA and viral mRNA, but it influences as well the subcellular distribution of the viral proteins. In fact, in untreated BDV-Vero cells, "N" and "P" viral proteins are localized both in the nucleus and in the cytoplasm [141]. With increasing concentration of CAF, both proteins disappeared from the cytoplasm and accumulated in the nucleus. Ribavirin treatment caused a comparable, but less drastic effect [7]. Since both "N" and "P" are basic constituents of viral ribonucleoparticles (RNP), their nuclear localisation is indicative of nuclear retention of viral RNP [7].

Taken together, all these data show that it is unlikely that CAF exerts its anti-BDV effect by interfering with the host cell machinery. However, the possibility cannot

be excluded that other unknown cellular pathways, important for the BVD replication, might be affected by CAF [7].

The activity of CAF has been reported also *in vivo,* where it has been described to inhibit BDV replication in the brain of infected rats, preventing persistent infection of the central nervous system as well as the development of clinical disease [7].

2.9.3 Amantadine

Amantadine sulfate is a well known substance which has been proved useful in the treatment and the prophylaxis of viral infections, in treating symptoms of Parkinson's disease, cocaine dependence and apathy in multiple sclerosis [43].
Besides these, a great number of other applications have been reported. For example, Amantadine is effective in post-herpetic neuralgia [48], in chronic fatigue syndrome of the multiple sclerosis patients [81], chronic hepatitis C [126], in the reduction of neuropathic pain [111] and in several others diseases [43].
Since 1966, it has been in use as an antiviral agent against influenza-A-virus infection. Its mechanism of action consists of an inhibition of the ion-channel activity of the M2 protein of the virus. It has been supposed that the drug acts by binding to a site in the open pore of the channel or altering its conformation through a distortion in one or more subunits [37, 107].
Amantadine was described as an antiviral active compound against BDV both *in vitro* and *in vivo* in a BDV-infected patient with bipolar depression [16]. Unfortunately, later studies couldn't confirm its effectiveness [55] neither with respect to the number of infected cells nor in the levels of BDV RNA or proteins [28]. The importance of BDV infection for psychiatric disorders in general and for depressive disorders is currently a matter of debate [66, 68, 84, 96], and consequently a possible therapy with Amantadine has often been hypothesized. Divergent results have been reported as to whether Amantadine has any effect at all on BDV infection *in vivo* or *in vitro* [28, 55, 131]. Many authors, however, do not point to the fact that all data were obtained with a laboratory adapted animal virus strain, whereas others [16] used a human virus strain. In the latter case, some study clearly support an antiviral effect of Amantadine [43].
On the other hand, Amantadine fails to induce a clearance of BDV from BDV-infected cells even if the results are not homogenous and susceptible for different interpretations: in fact, a recent study reports that no effect was detected on two cell lines (skin fibroblasts and astrocytic cell line F10 from Lewis rat and MDCK cells) while in one cell line (fibroblasts CRL 1405 from guinea pig), BDV virus was eliminated in one experiment with Amantadine at the concentration of 5 μg/ml [131]. Since the antiviral effect of Amantadine has so far not been reported to be dependent on the cell type used, the fibroblast CRL cell line might be extraordinarily sensitive to rather high concentrations of Amantadine. Alternatively

these results may indicate that Amantadine treatment of distinct cell lines yields a reduced intracellular virus titer without being able to eliminate the virus from those cells [84, 131].

In one of the newer studies, it has been reported as conclusion that permanent effects have never been ruled out entirely [43].

In vivo, Amantadine administered to rats had no influence on the time of onset of disease, neither on antiviral antibody titers, nor on virus titer in the brain, nor on the severity of neurological symptoms or encephalitic lesions [131].

2.10 Objectives

Considering the fact that no therapeutic approach has still been successively established against BD and the clinical significance of BDV infection has not been clarified, one aim of this study was to test different antiviral substances against BDV in persistently infected cells *in vitro*. Some of these compounds have already been reported as active against other viruses after chemical modification (β-lactoglobulin, albumin, lysozym, α-lactalbumin and bovine lactoferrin). Thus, it was interesting to test whether these compounds also possess antiviral activity against BDV. We were interested to study the antiviral activity of some substances whose antiviral properties against BDV were already described (Ribavirin and CAF), but their mechanisms were not yet assessed for sure. Another purpose of the study was to contribute to the controversial discussion about the antiviral activity of Amantadine. In order to appreciate a decrease of the infected cells attributable to an antiviral action, we tried to quantify the amount of infected cells when these substances were added to the culture medium during the incubation time.

Moreover in order to investigate if the viral diffusion and multiplication was mainly due to a cell to cell spread or rather to a transmission of the virus through the division and replication of infected cells, we established a cell labelling method able to mark the cell lineage.

3. Materials and Methods

3.1 Cell culture

MDCK (Madin Darby canine kidney cells ATCC Nr. CCL-34), MDCK BDV-infected cells (BDV isolate of the horse H1766) were a kind gift by Dr. S. Herzog, Institute of Virology, University of Giessen, Germany.

Non infected Vero (Vero 76 African green monkey kidney cells) cells were disposable at the Institute of Veterinary pathology, Vetsuisse-Fakultät der Universität Zürich, Switzerland.

Both cell lines (MDCK and Vero) were grown in Iscove's Mod. Dulbecco's Medium (Sigma®). To 500 ml medium, 50 ml 10% fetal bovine serum, inactivated at 60°C, 12.5 ml Hepes buffer (Sigma®), 5 ml L-Glutamine (Sigma®, 200 mM) and 5 ml Penicillin-Streptomycin solution (Sigma®) were added. The cells were washed twice with PBS (Invitrogen®) and splitted using trypsin (Trypsin-EDTA Solution, 1X, Sigma®) every 3-4 days, diluted 1:40 and maintained at 37 °C with 5% CO_2. Cells of both lines were trypsinized twice per week.

Vero cells were infected using a persistently BDV MDCK infected monolayer.

A confluent BDV persistently infected MDCK monolayer was split following the above described method. Then, the cell suspension was centrifuged at 800g for 10 min. The supernatant was discarded, the cells washed twice with PBS, centrifuged again and stored. The probe was frozen at –20 C° for an hour, then melted in warm running water. The procedure was repeated four times. The obtained solution, clear with white flakes, was filtrated twice with a 0.8-0.2 μm filter, and then added to the Vero cells after splitting.

Positive cells were first detected by indirect immunofluorescence after 2 weeks. Their number progressively increased for the next 3 weeks. After 4 weeks, ca. 98% of the cells were positive at the IIF.

3.2 Indirect Immunofluorescence (IIF) on cell cultures

The indirect immunofluorescence studies were performed on cell monolayers obtained from cells incubated in Lab-Tek® Chamber™ Slide System (from Nalge Nunc International) for three days (fig 1).

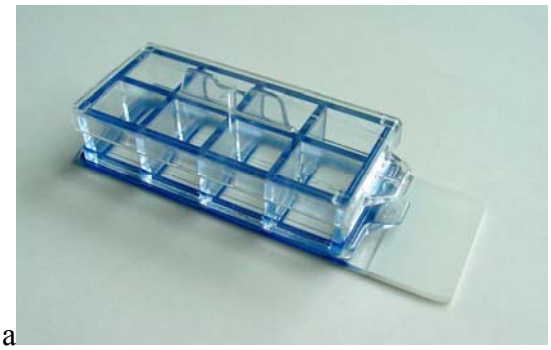
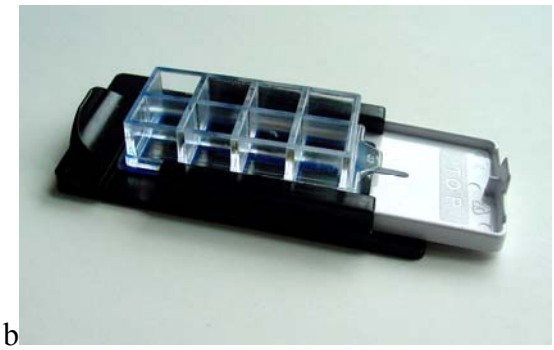
a  b 

Fig.1, a: Lab-tek® Chamber™ Slide System. Cells are incubated with medium and tested compounds for three days. The total amount of cell solution in every well was 400 µl (containing 4 x 10^4 MDCK cells or 6 x 10^6 Vero cells); b: removal of the wells wall after the incubation. The slide was then analysed by immunofluorescence procedure.

In each well of the Lab-tek® (9x8 mm in size) 100 µl of a cell solution was dropped (4x10^5 /ml MDCK cells 6x 10^7/ml Vero cells). The volume in every well was brought to 400 µl by adding medium. Cells were incubated only with medium as negative control and with progressively higher concentrations of the substance to test, as reported in the scheme of figure 2. Every experiment was carried out in double, and each row of the Lab-Tek® wells (1 to 4 and 5 to 8) was considered an independent unit.

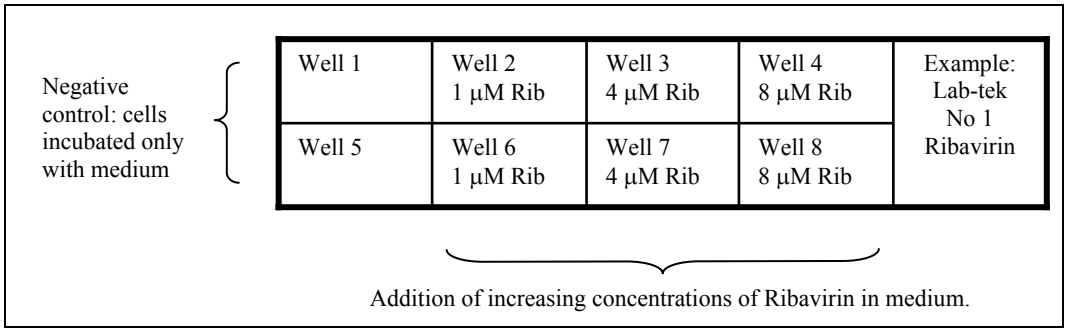


Fig 2: layout of a Lab-tek® slide: placing of the negative control and progressive increasing concentrations of Ribavirin.

After the incubation, medium was discarded, the plastic wells were removed and the monolayer was washed twice with PBS. The monolayers on the slide were fixed for 20 min with –20°C Aceton at room temperature.

Viral antigen was stained by adding 100 µl of 1:2000 diluted anti BDV-chicken serum (obtained from experimentally BDV infected chicken, gently gifted from Dr. S. Herzog, Institute of Virology, University of Giessen, Germany) in each well. The slide was then incubated for one hour at 37 °C and washed with PBS. 100 µl of 1:200 diluted labelled antibody (Alexa Fluor® 488 goat anti-chicken IgG (H+L) were applied on every well and incubated again for one hour at 37 °C. They were washed with PBS and dipped 5 seconds in 1:10000 diluted Evan's Blue as counter stain. The slides were extensively washed again with PBS, covered with cover glasses and the interposition of few drops of Glycerin (Kaisers Glyceringelatine, Merck) diluted 1:10 with PBS. Finally the slides were analysed by fluorescence microscopy (Mikroskop LEICA DMLS) at a wavelength of 450-490 nm (filter I3 Leica).

3.3 Chemical modification of proteins

The following proteins were chemically modified through reaction with 3-HP (Aldrich) as reported in the literature [102]: bovine serum albumin (BSA, Fluka®), lysozyme from chicken egg white (Fluka®), α-lactalbumin (Sigma), β-lactoglobulin (Sigma®) and bovine lactoferrin (gently gifted from Dr. Recio, Madrid).

Briefly, 160 mg of the purified proteins were dissolved in 5 ml of 0.1 M Na-phosphate buffer pH 8.5. 3-HP solution was added in eight aliquots of 200 µl (125 mg/ml 3-HP dissolved in dimethylsulfoxyde) at 12 min. intervals. The solution was maintained at pH 8.5. After 1 hour incubation at 25 °C, the mixture was dialysed against 500 ml PBS pH 7.4 (Dialysation membranes Spectra/Por® MWCO: 6000-8000). Protein concentration was determined photometrically as described [102].

Antiviral activity of Lactoferrin, β-lactoglobulin, albumin and lysozym had been tested both before and after chemical modification.

α-lactoalbumin had been tested only after chemical modification.

3.4 Antiviral chemical compounds

All the tested chemical compounds were purchased from Sigma®:
Ribavirin (1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide), CAF (Cytosine β-D-arabinofuranoside) and Amantadine (Amantadine hydrochloride).

3.5 Tests on persistently BDV-infected cells

A confluent BDV infected cells monolayer was split as reported in 3.1 and the cells suspension (ca. 2 x 10^6 cells/ml) of cells was diluted with medium (1:5 for MDCK and 1:3 for Vero cells).
Then, 100 µl of cell solution was applied in every well of the Lab-Tek® and 300 µl medium were added. Then, the different concentrations of tested substances were added in every well, and the cells were incubated for three days at 37°C. The third day, the medium was discarded, and the Lab-tek® processed according to the IIF method described above.
In order to standardise the number of infected cells and their variation, we considered 5 fields on every well in predetermined placing: upper left, upper right, lower left, lower right and in the middle of the well. On each of these 5 fields, the number of positive cells (out of 100 cells) was counted. When the monolayer was somehow damaged or not homogenous, the predetermined placings were slightly rearranged, so that a cell count could be anyway performed.
The obtained data were statistically processed by the Bonferroni's multiple comparison test, using the application program 'Prisma'. Results showing a $P<0.05$ were considered statistically significant.

3.6 Tests on BDV-infected and non infected cells

Confluent monolayers of non infected and BDV persistently infected cells were split following the method described. The cell suspension (ca. 2 x 10^6 cells/ml) was diluted with medium (1:5 for MDCK cells, 1:3 for Vero cells). Then BDV-infected and non infected cells of the same cell line were mixed in a ratio of 1:400. After intensive

mixing, 100 µl of this solution were dropped in each well of the Lab-Tek® Chamber Slide™. Solution of different concentrations of substances or proteins to test were added in the incubation chambers and the final volume was brought to 400 µl with medium and incubated for three days at 37°C.

One focus forming unit (FFU) was defined as the amount of virus required to form a cluster of several infected cells in the monolayer, identified by IF [55]. The number of fluorescent cell foci and the number of fluorescent cells per focus were reported. In the contest of the study, the expression FFU was used to indicate a cluster of BDV infected cells.

Data obtained were statistically processed by the Bonferroni's multiple comparison test, as described above.

3.7 Cell labelling by CMTPX

In our investigation, both the number of FFU and the number of fluorescent cells per FFU were evaluated. In order to investigate if a reduction of the number of infected cells was due to a cytostatic or cytotoxic effect of the tested substances, or rather to an inhibition of the virus spread from cell to cell, we developed a procedure which uses the cell marker CMTPX (CellTracker Red CMTPX Molecular Probes).

CMTPX, as another cells tracker widely documented (CFSA) to recognize viable cell progeny [6, 82, 88]. Moreover, and differently from other cell markers, CMTPX has the property not to interfere with the green Alexa fluorochrom staining. It is visible when excited at 613 nm. This property makes this compound very useful to investigate the viral spread.

CMTPX is a cell tracker dye that is retained in living cells through several generations. It is inherited by daughter cells after cell fusion, equally apportioned between the two daughter cells and it is not transferred to adjacent cells in a population. When incubated with living cells, it passes freely through cell membranes, but once inside the cell, it is transformed into a cell-impermeant reaction product [1]. CMTPX contains a chloromethyl group that reacts with thiol groups present in proteins through a glutathione S-transferase-mediated reaction. In this way CMTPX is transformed into a cell-impermeant fluorescent dye – thioether adduct. Excess unconjugated reagent passively diffuses to the extracellular medium. The fluorescent signal, as in other cell markers like carboxyfluoroscein, progressively halves with each mitosis [45]. CMTPX yields a brightly red cytoplasmatic staining when examined by fluorescence microscopy (LEICA DMLS) at the wavelength of 575 nm (filter G/R Leica). However, CMTPX doesn't emit any appreciable light when excited in the range of 450-490 nm (Alexa fluorochrom excitation spectrum).

The number and the size of every FFU were reported as previously described. In our study, we investigated the number of daughter cells per FFU (CMTPX labelled and BDV-infected cell), the number of new infected cells per FFU (CMTPX non labelled, BDV-infected cells) and the number of FFU constituted only by daughter cells .

Data were statistically processed by the Bonferroni's multiple comparison test, as described above.

Cells were labelled with CMTPX as follows:

BDV infected cells were trypsinized and 980 µl of cells suspension were incubated with 20 µl CMTPX 1mM for one hour at 37 °C. Every 15 min the probe was gently stirred for about 15 sec. The probe was then centrifuged (5 min, 500 g), the supernatant discarded, replaced with the same volume of medium and incubated 30 min at 37 °C. The cells were centrifuged once again, medium was discarded, and the cells washed twice with PBS. Finally, the same volume of medium was replaced in order to restore the original cell concentration. Cells were diluted with medium (1:5 for MDCK and 1:3 for Vero) and then incubated for three days together with non infected cells (ratio 1:400). The compounds to be tested were added into the assay mixture as previously described. Cells were then investigated using a fluorescence microscope. Evan's blue counter stain was not used because it produces interferences with CMTPX.

At the concentration used, CMTPX did not produce any toxic effect on the cells: they were grown until confluence after three days incubation without any change in shape, morphology or density on optical control.

When the cell monolayer was analysed at 450 nm (filter I3 Leica), only the infected cells were visible, organized in FFU. Using an excitation light of 613 nm (filter G/R), both fluorochrom Alexa (and then BDV infected cells) and CMTPX appeared lightened.

3.8 Statistical analysis

The data obtained were statistically processed through the Bonferroni's multiple comparison test, using the application program 'Prisma'. Results showing a $P<0.05$ were considered significant. In the graphics reported, the box represents the interquartile range, the whiskers represent the maximum and the minimum value observed and the bold line represents the median. Additionally, tables are reported in the annex showing more in detail mean and standard deviation of every group of data.

4. Results

4.1 Cell culture

BDV non infected cells assessed with IIF appeared as uniform dark monolayer (fig. 3). Only at the highest magnification (objective 40x) it was possible to distinguish the nucleus from the cytoplasm.

BDV infected cells showed multiple, round, bright yellow inclusions in the nucleus (fig.4, a and b). The cytoplasm appeared dark, but the cell borders were almost quite easily visible. With the dilutions and the incubation time described, cells grew on the wells confluently. In a persistently BDV infected monolayer the percentage of cells positive was 85 to 100% for MDCK cells and 90 to 100% for Vero cells.

When infected cells were incubated with non infected cells, IIF positive cells were grouped in clusters, indicated as focus forming unit (FFU) and surrounded by negative cells (fig.5 and 6). The number and the size of the FFU (expressed as the number of infected cells per FFU, see 3.6) were counted and statistically analysed.

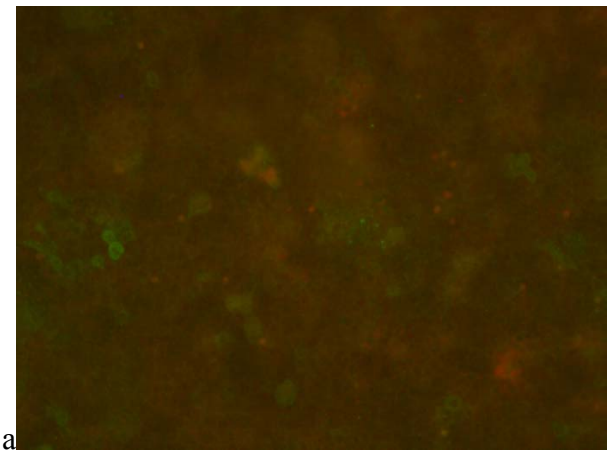
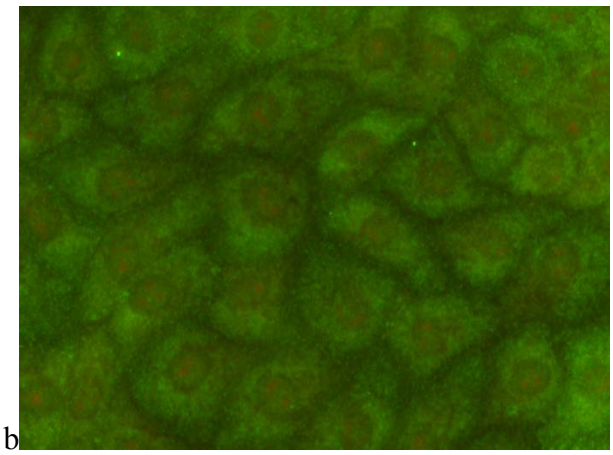
a  b 

Fig. 3: MDCK non infected monolayer, 10x objective (a) and 40x objective (b), immunofluorescence microscope, filter I3 Leica.

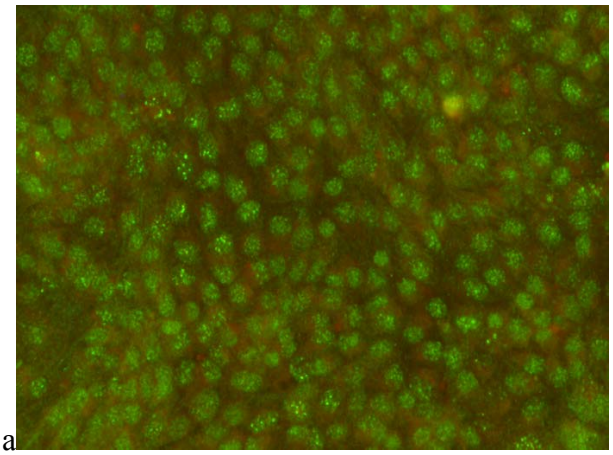
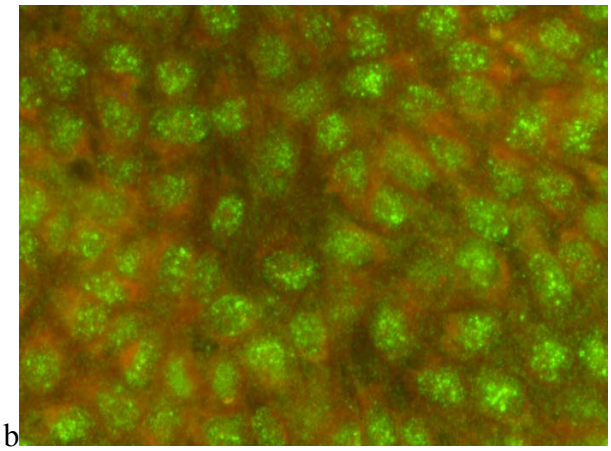
a  b 

Fig. 4: MDCK BDV persistent infected monolayer, 20x objective (a), 40x objective (b) immunofluorescence microscope, filter I3 Leica.

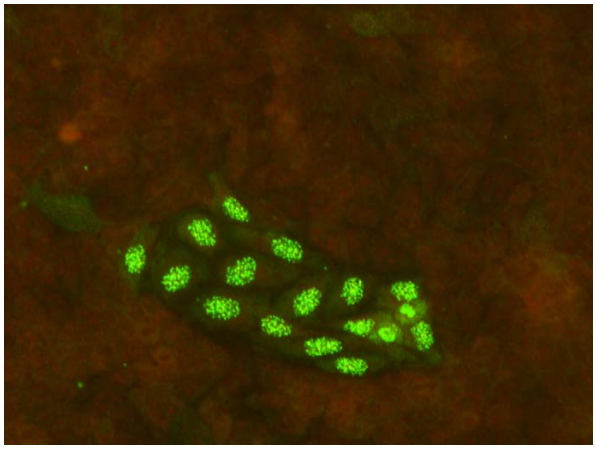
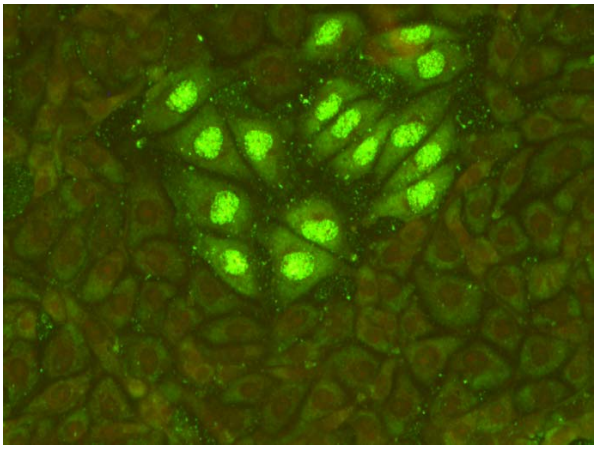
 

Fig. 5 and 6: MDCK BDV infected cells organized in FFU, 40x objective, immunofluorescence microscope, filter I3 Leica.

4.2 Antiviral activity of the native and modified proteins on BDV-infected MDCK cells

The proteins lactoferrin, β-lactoglobulin, albumin, lysozyme were tested for antiviral activity both before and after chemical modification.
3-HP was also tested as a negative control at the concentrations of 10, 20, 200 μM and 2, 20 mM. Since the cell monolayer appeared complete after the three days incubation and no morphological changes in density, shape and size were detected, the possibility of an intrinsic cytotoxicity was ruled out.
All the proteins were tested on MDCK BDV persistently infected cells and on MDCK BDV infected and non infected cells incubated together.

4.2.1 Bovine lactoferrin

Native bovine lactoferrine was tested at the concentration of 3.0, 6.0 and 12.0 μM. Lactoferrin did not show any effect on cell growth and/or any cytotoxic effect like changes in cells morphology, size or shape, or density. Moreover, the cell monolayer appeared confluent and homogeneous after the standard incubation time.
The percentage of infected cells showed a slight reduction when incubated with all the three concentrations of lactoferrin compared to the control (incubated only with medium), but without statistical significance (data not shown). The number and the size of FFU did not show any change.
After chemical modification, 3-HP lactoferrine was tested at the concentration of 0.3, 0.6, 1.25 μM and in all tests a marked inhibition of the cells growth was present, suggesting a strong cytotoxicity of this compound.

4.2.2 β-Lactoglobulin

β-Lactoglobulin was tested at the following concentrations: 3.0, 6.0, 12.0, 25, 50, 100 μM. Neither the percent of infected cells nor the number or the size of the FFU showed any change.

3-HP β-lactoglobulin had an inhibitory effect on cell replication at concentrations higher than 1 μM. In this case, the monolayer obtained was not homogeneous, and the cell density was reduced. The count of the number of infected cells was not reliable. Anyway, no change in the percentage of infected cells or in the number and in size of the FFU was detectable.

4.2.3 Albumin

Albumin and 3-HP albumin were tested at the following concentration: 0.5, 1, 5 and 10 μM. Both the substances exerted a strong cytotoxic effect on the cell growth.

4.2.4 Lysozyme

Lysozyme was tested at the following concentrations: 3.0, 6.0, 12.0, 25, 50, 100 μM
It did not show any antiviral activity. 3-HP Lysozyme strongly inhibited the cell growth at all the concentrations of 1, 5, 10, 20, 50 μM.

4.2.5 3-HP α-lactalbumin

3-HP α-lactalbumin was tested at the following concentrations: 0.5, 1, 5, 10 μM.
No significant reduction of the percent of BDV infected cells. When both infected and non infected cells were incubated with 3-HP α-lactalbumin, a slight inhibition on the cell growth at the concentration of 5 μM was noticed. This could suggest a higher selective sensitivity of the non infected cells compared to the BDV infected cells to the 3-HP α-lactalbumin.
With concentrations lower than 5 μM, no reduction in the number or size of FFU was detectable.

4.3 Anti BDV activity of chemical antiviral substances

4.3.1 Antiviral activity on BDV persistently infected cells:

4.3.1.1 Ribavirin

The percentage of MDCK infected cells incubated with different concentrations of Ribavirin (1, 4, 8 μM) progressively decreased with increasing the concentrations of Ribavirin (fig.7a). Statistical significance was detected for 0 vs. 8 μM Ribavirin ($P<0.01$).
Similar results were obtained with Vero cells (fig.8a): statistically significant reduction in the percentage of infected cells was detected for 0 vs. 8 μM Ribavirin ($P<0.05$).

4.3.1.2 CAF

The percentage of MDCK infected cells decreased when incubated with CAF. In particular, the differences for 0 vs. 1 μM ($P<0.05$) and for 0 vs. 4 and 0 vs. 8 μM ($P<0.01$) of CAF were statistically significant (fig.7b)
When persistently infected Vero cells were incubated with CAF (1, 2 or 4 μM), no statistically significant change in the percentage of infected cells could be noticed (fig.8b)

4.3.1.3 Amantadine

Amantadine was tested at the following concentrations: 0, 16, 32, 64 μM. The decrease of the percentage of MDCK infected cells was statistically significant only between the 0 and 64 μM ($P<0.01$) (fig.7c).
No statistically significant change has been detected in the same tests using Vero cells (fig.8c).

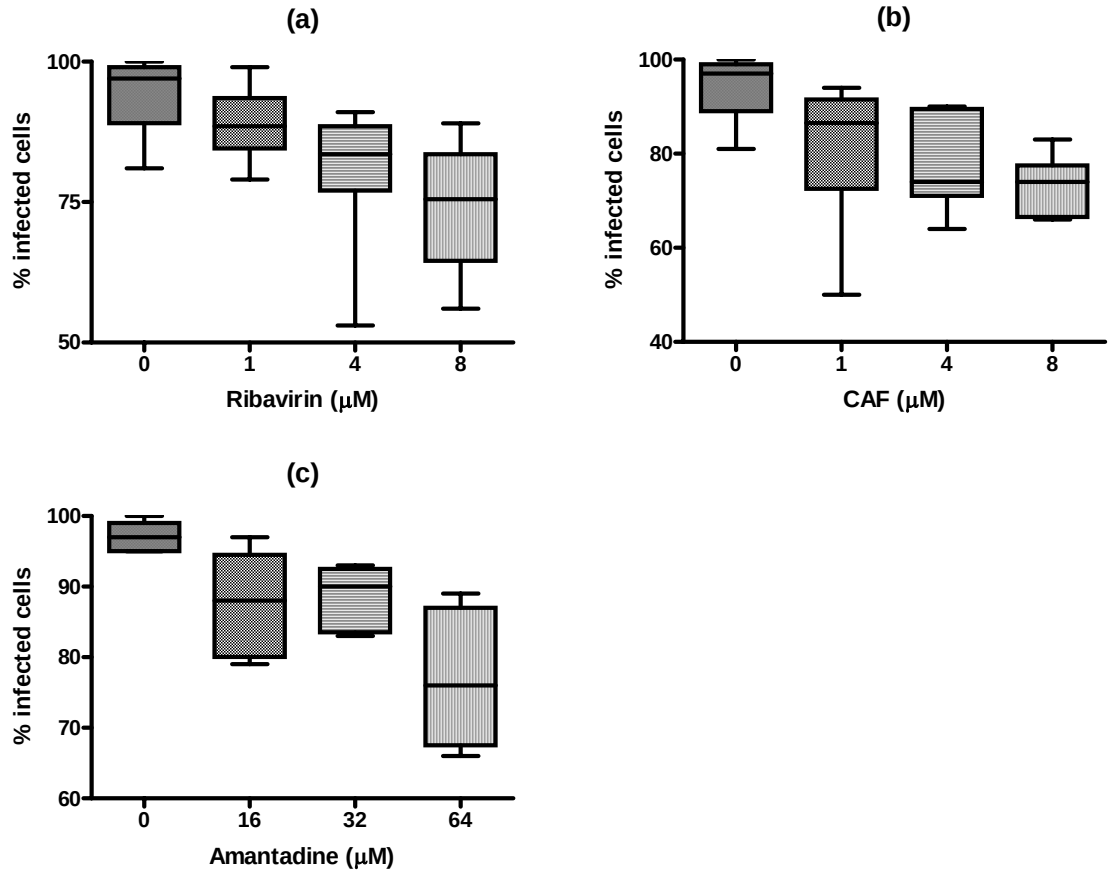


Fig. 7: (a) Effect of Ribavirin on BDV persistently infected MDCK cells. The percentage of BDV infected cells is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.01$) for 0 vs. 8 μM. (b) Effect of CAF on BDV persistently infected MDCK cells. The percentage of BDV infected cells is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.05$) for 0 vs. 1 μM, $P<0.01$ for 0 vs. 4 and 0 vs. 8 μM. (c) Effect of Amantadine on BDV persistently infected MDCK cells. The percentage of BDV infected cells is reported as a function of the concentration of Amantadine. Statistically significant difference was detected ($P<0.01$) for 0 vs. 64 μM.
In the graphics the box represents the interquartile range, the whiskers represent the maximum and the minimum value observed and the bold line represents the median.

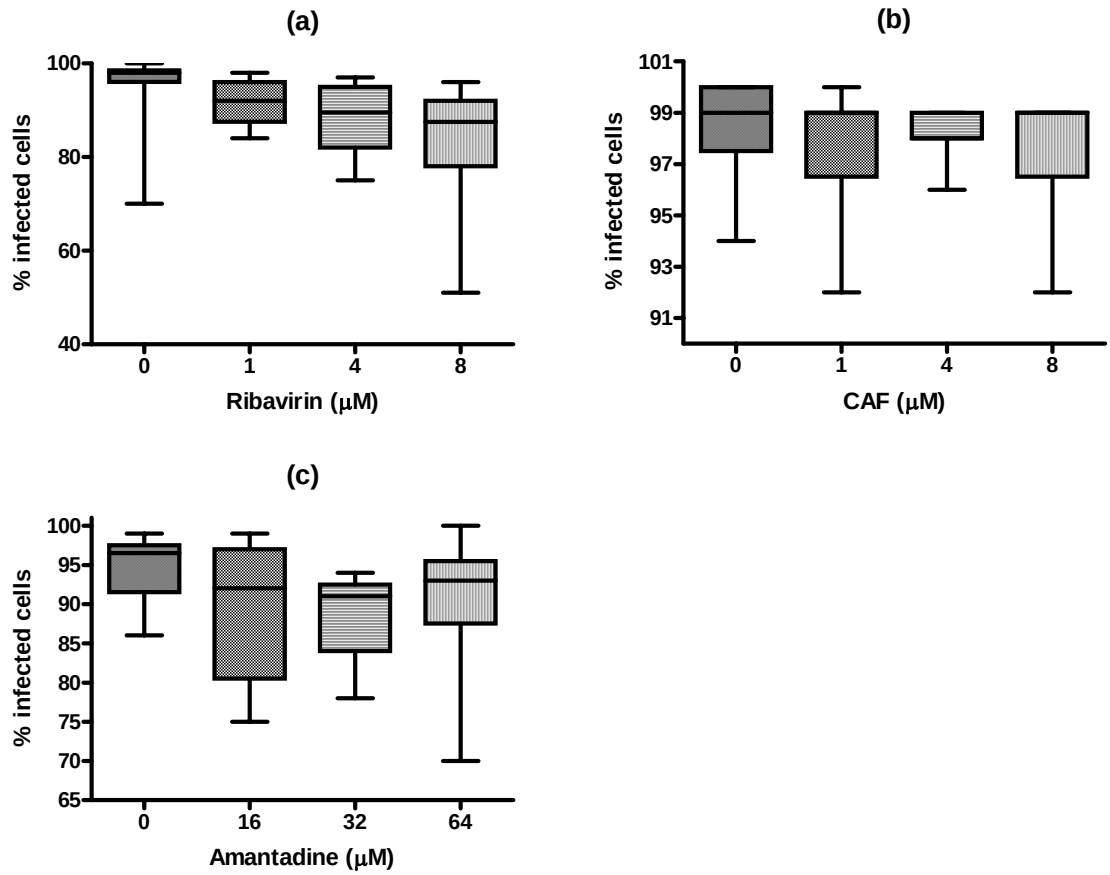


Fig. 8: (a) Effect of Ribavirin on BDV persistently infected Vero cells. The percentage of BDV infected cells is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.05$) for 0 vs. 8 μM. (b) Effect of CAF on BDV persistently infected Vero cells. The percentage of BDV infected cells is reported as a function of the concentration of CAF. No statistically significant difference was detected.
(c) Effect of Amantadine on BDV persistently infected Vero cells. The percentage of BDV infected cells is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. See fig. 7 for key.

4.3.2 Antiviral activity on size and number of Focus Forming Units (FFU)

To investigate the anti BDV activity of some antiviral substances on BDV infected and non infected cells, the number of FFU and their size (that was expressed as the number of infected cells per FFU) was evaluated.

4.3.2.1 Ribavirin

Ribavirin concentrations tested on MDCK cells in the assays were 1, 4, 8 μM. At these concentrations of Ribavirin, the number of FFU remained unchanged, their size decreased significantly following a dose dependent course (fig.9 a and b). A statistically significant reduction in the size of the FFU was detectable ($P<0.001$) when the results obtained for the following concentration were compared for 0 vs. 4, 0 vs. 8, 1 vs. 4 and 1 vs. 8 μM.

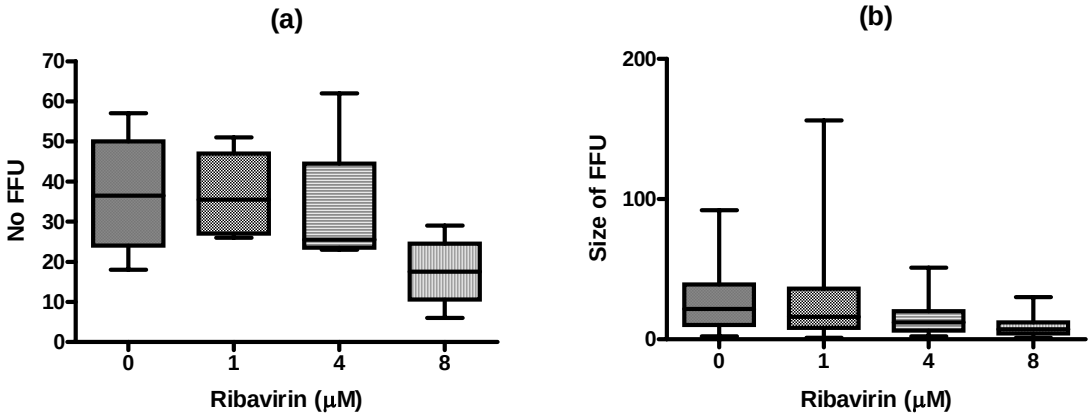


Fig. 9: Effect of Ribavirin on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.001$) for 0 vs. 4, 0 vs. 8, 1 vs. 4 and 1 vs. 8 μM. See fig. 7 for key.

The same concentrations of Ribavirin did not lead to any statistically significant change neither in the number nor in the size of FFU in Vero cell tests (fig.10 a and b).

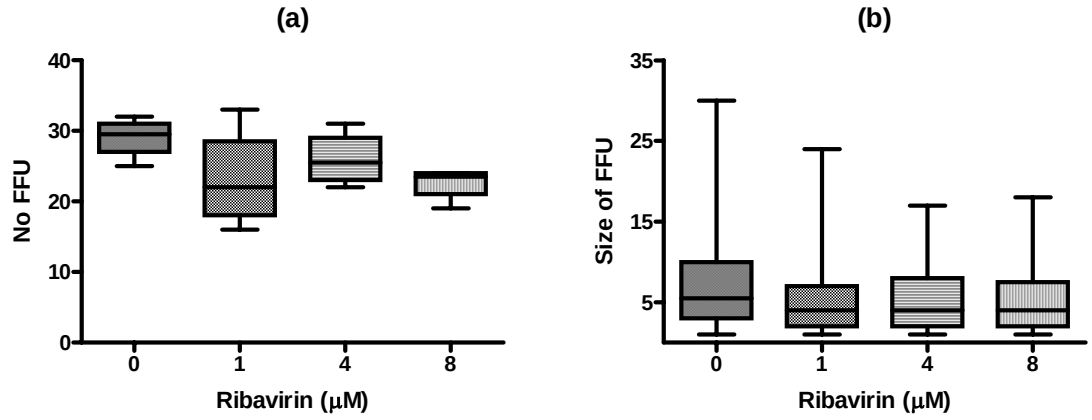


Fig. 10: Effect of Ribavirin on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. See fig. 7 for key.

4.3.2.2 CAF

On MDCK cells, CAF was tested at concentrations of 1, 4, 8 μM. The decreased number of FFU is statistically significant ($P<0.01$) for concentration of 0 vs. 4 and 0 vs. 8 μM.

At the same time also the size of the FFU decreased following a dose dependent course. The size of FFU differed highly statistically significant ($P<0.001$) for the following concentrations: 0 vs. 1, 0 vs. 4, 0 vs. 8, 1 vs. 4 and 1 vs. 8) (fig. 11 a and b).

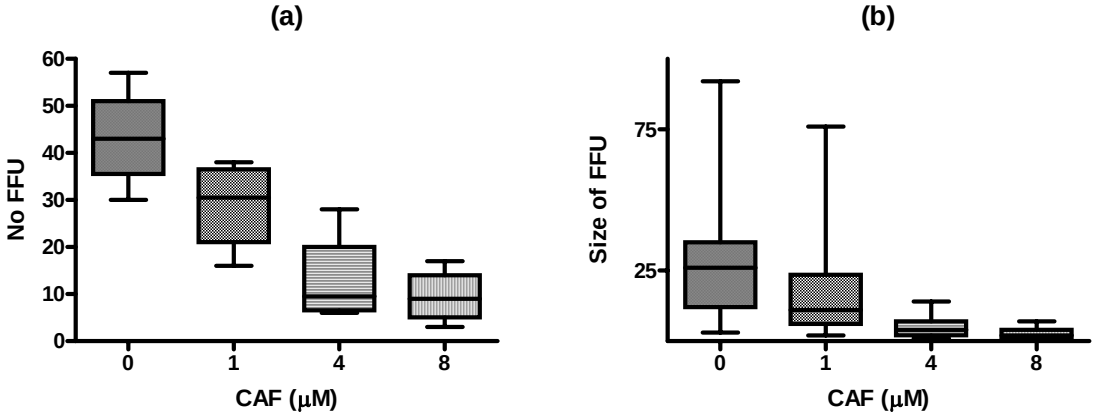


Fig. 11: Effect of CAF on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.01$) for 0 vs. 4 and 0 vs. 8 μM. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.001$) for 0 vs. 1, 0 vs. 4, 0 vs. 8, 1 vs. 4 and 1 vs. 8 μM. See fig. 7 for key.

On Vero cells, CAF was tested at concentrations of 1, 2, 4 μM. For higher concentrations, cells density appeared reduced and the monolayer did not grow on all the available surface of the well. The number of FFU was highly significantly decreased (P<0.001), in presence of CAF, for concentrations of 0 vs.1, 0 vs. 2 and 0 vs. 4 μM.

Similar results were obtained considering the size of the FFU (P<0.001) for concentrations of CAF 0 vs.1, 0 vs. 2 and 0 vs. 4 μM (fig.12 a and b). No statistically significant differences between the tested concentrations of CAF regarding the number and the size of the FFU were noted

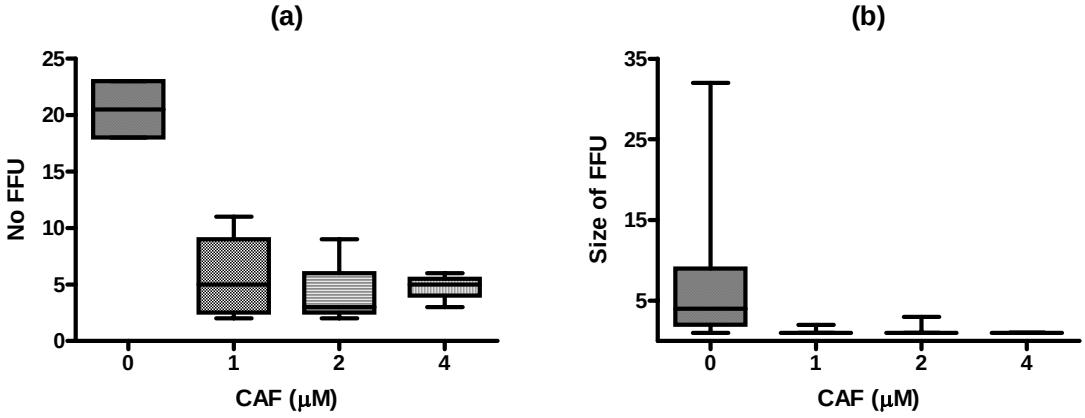


Fig. 12: Effect of CAF on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of CAF. Statistically significant difference was detected (P<0.001) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of CAF. Statistically significant difference was detected (P<0.001) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. See fig. 7 for key.

4.3.2.3 Amantadine

No difference was detected neither in the number of FFU nor in their size at concentrations of 16, 32, 64 μM of Amantadine when compared with the negative control on both MDCK and Vero the cell lines (fig.13 a and b and 14 a and b).

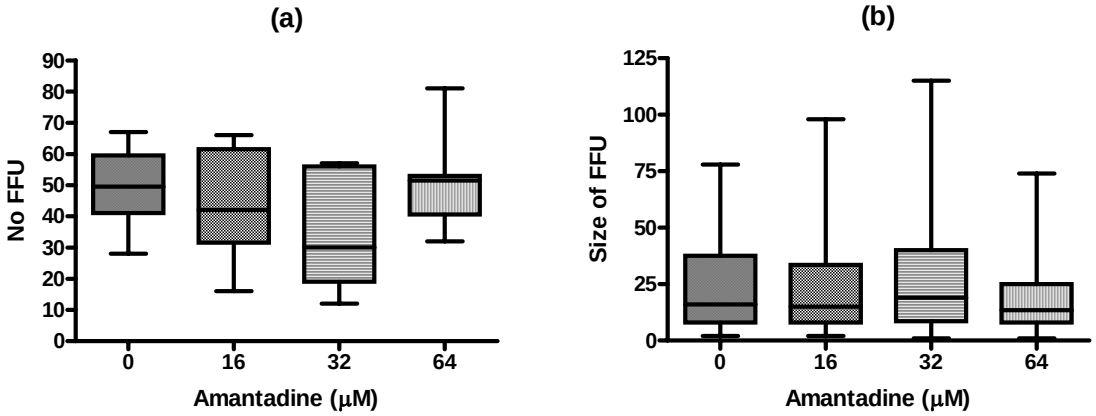


Fig. 13: Effect of Amanatdine on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. See fig. 7 for key.

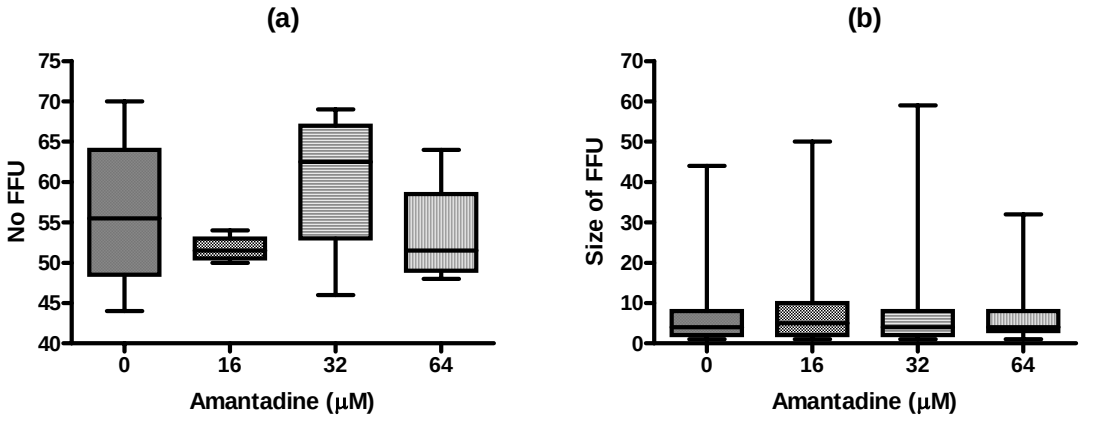


Fig. 14: Effect of Amanatdine on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (b) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. See fig. 7 for key.

4.4 Progression of infection, use of CMTPX as cell marker.

Tests using CMTPX as cell marker were performed on both cell lines. As described in 3.7, BDV infected and CMTPX labelled cells were incubated with BDV non infected cells.

With the use of CMTPX as cell label and reading the slides using a fluorescence microscope, filter G/R Leica, both stainings were clearly visible: Alexa immunofluorescence as bright yellow nuclear inclusions and CMTPX label as red, diffuse cytoplasmatic staining (fig.15 a and b). In the following discussion, the definition of 'CMTPX positive' cell indicates a cell labelled with CMTPX, that was a daughter cell coming from the original BDV persistently infected monolayer.

Therefore, there were four possible cell conditions:

- double negative cell;
- CMTPX positive and BDV-negative cell (indicated as CMTPX positive);
- CMTPX negative and BDV-positive (indicated only as infected cell);
- double positive cell (BDV positive and CMTPX positive).

The composition of every FFU was investigated and reported. In detail, there were three possible types of FFU were observed:

- FFU where all the cells were double positive (it means that all the infected cells were coming from the same original CMTPX incubated cells) (fig.16 a and b);
- FFU where the infected cells (but CMTPX negative) cells were more numerous than the CMTPX positive cells (fig.17 a and b). These represent the cells that have been infected during incubation by the virus spread from cell to cell or from free virus in the medium;
- FFU where the CMTPX positive (but non infected) cells were more numerous than the infected cells (fig.18 a and b). In this case, the virus has not been successfully transmitted through the cell replication or the cells come from negative cells present in the persistently infected monolayer.

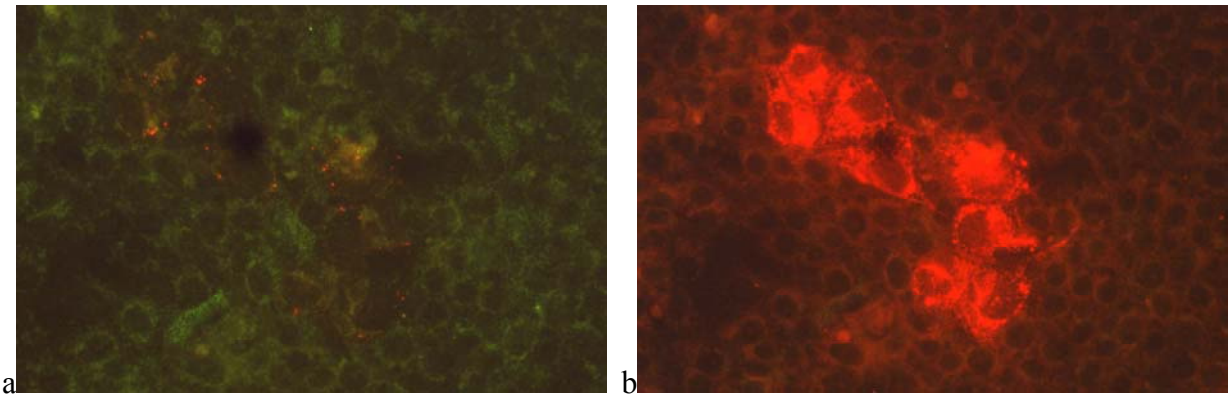


Fig. 15 a : In a MDCK BDV negative monolayer, some cells were incubated with CMTPX (in a ratio of 1:400). With the filter I3 Leica (a), CMTPX staining was not visible. With the filter G/R Leica (b), CMTPX labelled cells are clearly visible. 40x objective.

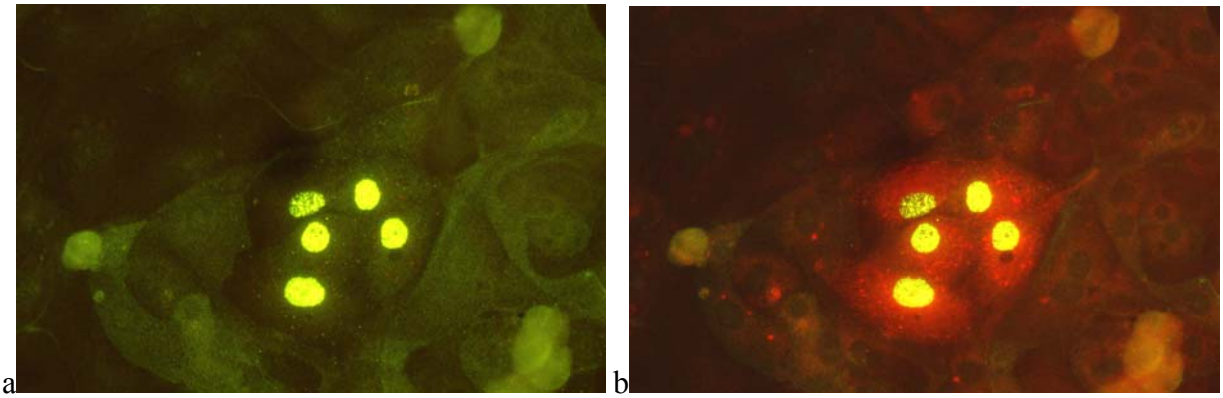


Fig. 16 a and b: BDV infected Vero cells organized in FFU, positive at indirect immunofluorescence. Alexa staining is visible using the filter I3 Leica (a) as well as using the filter G/R Leica (b). CMTPX staining is only visible using the filter G/R Leica. In this case all the cells composing the FFU are daughter cells from the original BDV infected monolayer CMTPX labelled. 40x objective.

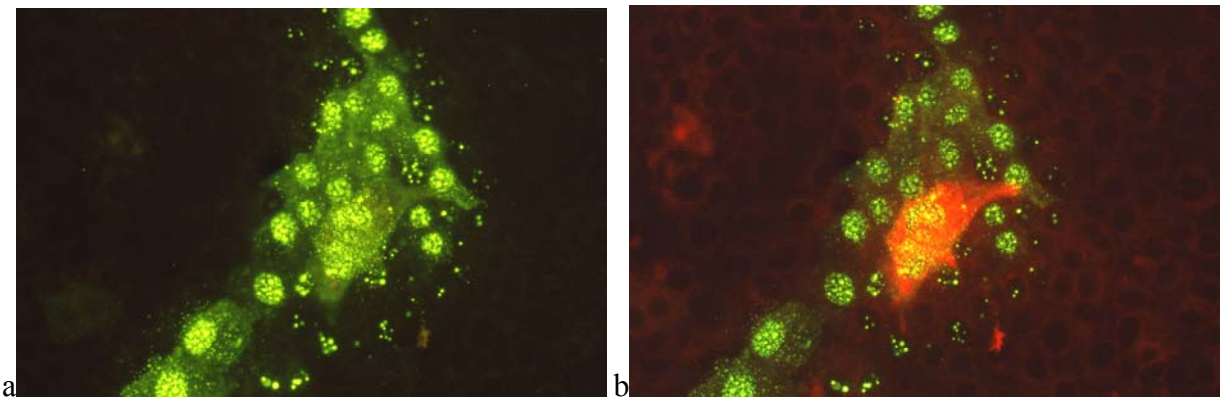


Fig. 17 a and b: BDV infected MDCK cells organized in FFU, positive at the indirect immunofluorescence. Fluorochrom Alexa staining is visible using the filter I3 Leica (a) as well as using the filter G/R Leica (b). CMTPX staining is only visible on the filter G/R Leica. In this case, only two of the BDV positive cells composing the FFU come from the cell linage labelled with CMTPX. BDV positive cells but not CMTPX labelled are interpreted as infected probably due to virus spread from cell to cell. 40x objective.

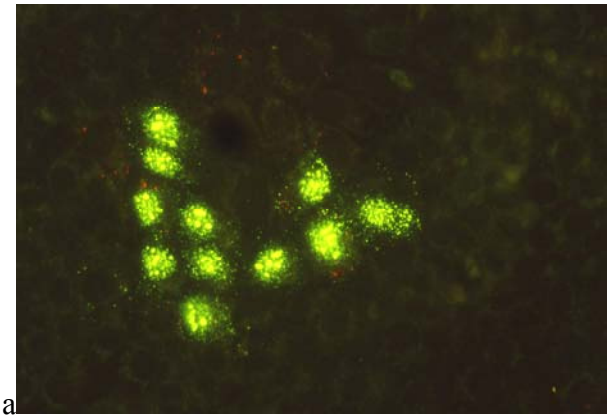
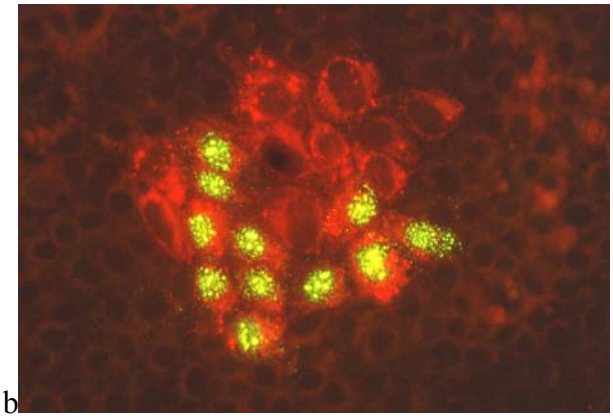
a  b 

Fig. 18 a and b: BDV infected MDCK cells organized in FFU, positive at the indirect immunofluorescence. Fluorochrom Alexa staining is visible using the filter I3 Leica (a) as well as using the filter G/R Leica (b). CMTPX staining is only visible on the filter G/R Leica (b). In this case, the CMTPX labelled cells are more numerous than the BDV infected cells. These are interpreted as cells coming from the original BDV persistently infected monolayer that didn't transmit the infection through the cell multiplication. 40xobjective.

In order to show the composition of every single FFU, the proportion of the daughter cells in every FFU was related to the number of infected cells. It was expressed as the number of double positive cells divided by the number of BDV infected cells in the correspondent FFU.

In the same way, the number of newly infected cells in every FFU was reported as the number of BDV infected but CMTPX-negative cells divided by the total number of BDV infected cells in the correspondent FFU.

Moreover, the proportion of FFU composed of only double positive cells was reported, calculated as the ratio between the number of FFU composed of only double positive cells divided by the total number of FFU in the well.

4.4.1 Ribavirin

On MDCK cells the same concentrations of Ribavirin of the previous experiments (see 4.3.2.1) were tested (1, 4, 8 μM) and no reduction in the number of FFU was assessed (fig. 19a).

The number of FFU only composed of double positive cells showed a constant increase, statistically significant at the concentration of 8 μM ($P<0.05$), compared to the negative control (fig.19b).

A reduction in the size of the FFU was detected, statistically significant: $P<0.01$ for 0 vs. 4, $P<0.05$ for 1 vs. 4 μM. $P<0.001$ for 0 vs. 8 and 1 vs. 8 μM (fig.19c).

The number of new infected cells per FFU significantly and progressively decreased with the addition of Ribavirin. $P<0.05$ for 0 vs. 8 μM and 1 vs. 4 μM. $P<0.01$ for 1 vs. 8 μM (fig.19d).

The number of daughter cells per FFU was not dependent on the Ribavirin concentration present in the assay (fig.19e).

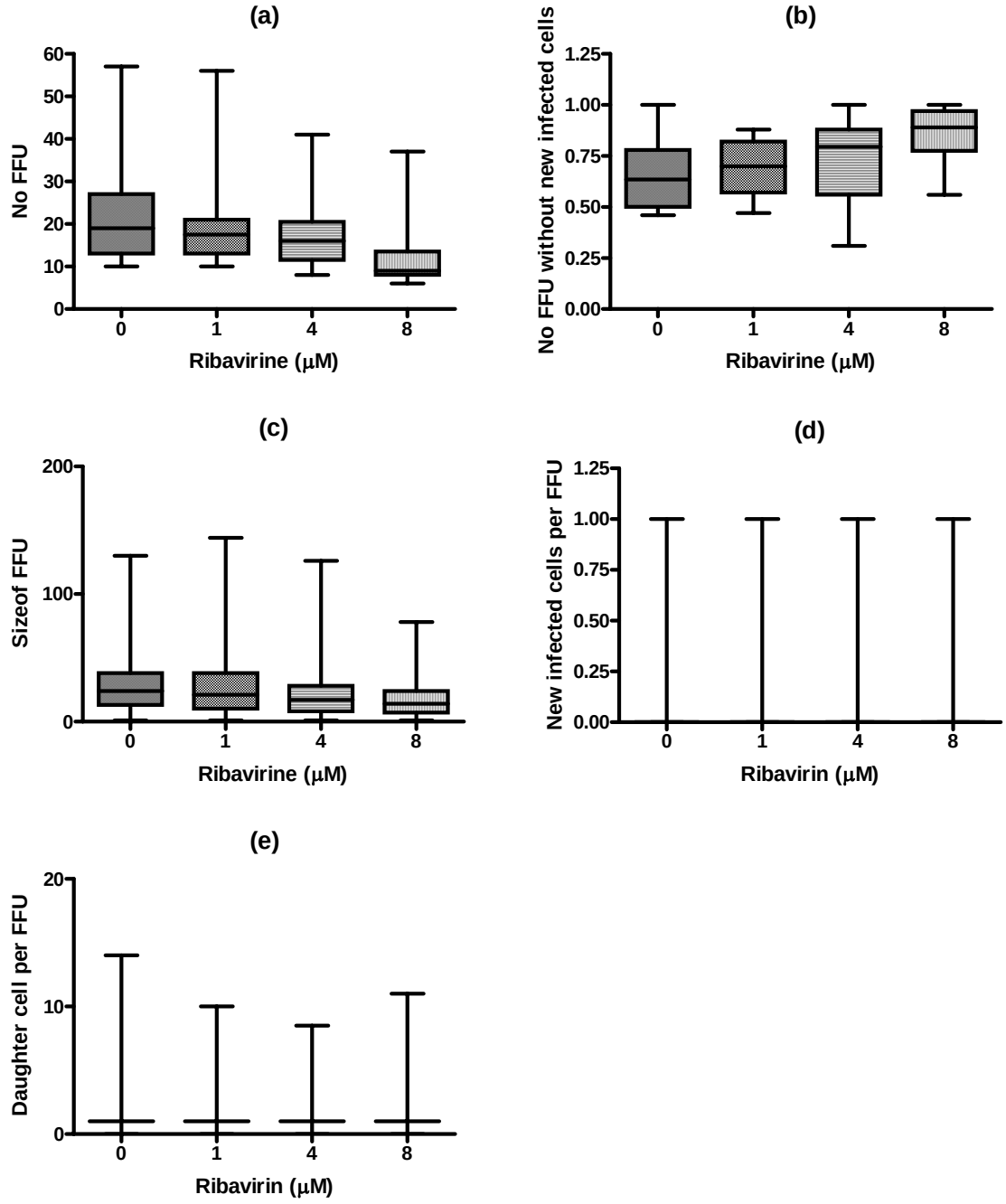


Fig. 19: Effect of Ribavirin on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (b) The number of FFU without new infected cells is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.05$) for 0 vs. 8 μM. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.01$) for 0 vs. 4; $P< 0.001$ for 0 vs. 8 and 1 vs. 8; $P<0.05$ for 1 vs. 4 μM. (d) The number of new infected cells per FFU is reported as a function of the concentration of Ribavirin. Statistically significant difference was detected ($P<0.05$) for 0 vs. 8, 1 vs. 4; $P<0.01$ for 1 vs. 8 μM. (e) The number of daughter cells per FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected.See fig. 7 for key.

When Ribavirin was tested on BDV infected and non infected Vero cells, the number and the size of the FFU as well as the number of daughter cells per FFU remained unchanged (fig. 20 a, b, c, e)

The only statistically significant difference was observed in the decreased number of new infected cells per FFU ($P<0.05$) for 0 vs. 1 μM (fig. 20 d).

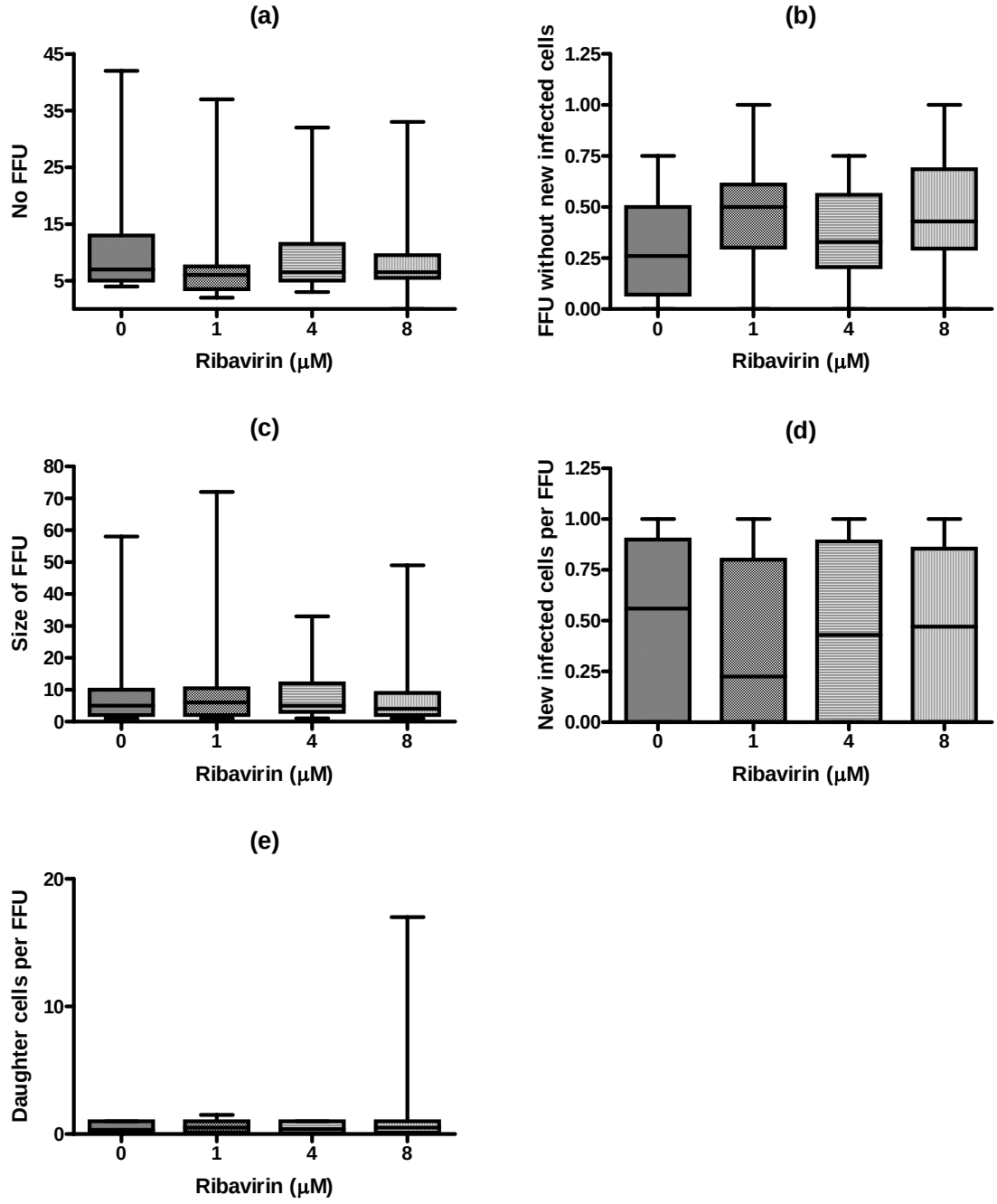


Fig. 20: Effect of Ribavirin on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (b) The number of FFU without new infected cells is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected. (d) The number of new infected cells per FFU is reported as a function of the concentration of Ribavirin. Statistical significant difference was detected ($P<0.05$) for 0 vs. 1 μM. (e) The number of daughter cells per FFU is reported as a function of the concentration of Ribavirin. No statistically significant difference was detected.See fig. 7 for key.

4.4.2 CAF

CAF was tested on BDV infected and non infected MDCK cells at the concentrations of 1, 2, 4 μM.

The number of FFU showed a statistically significant decrease ($P<0.01$ for 0 vs. 4 μM) (fig. 21 a).

The number of FFU composed only of daughter cells showed a progressive, significant enhance: $P<0.01$ for negative control vs. 1 and vs. 2 μM; $P<0.001$ in 0 vs. 4 and 1 vs. 4 μM (fig. 21b).

Also the size of the FFU decreased with statistical relevance: $P<0.01$ for 1 vs. 2 μM, $P<0.001$ for 0 vs. 1, 0 vs. 2, 0 vs. 4 and 1 vs. 4 μM of CAF (fig.21 c).

The number of daughter cells significantly decreased in presence of 1 μM of CAF in assay ($P<0.05$). The number of new infected cells was as well strongly reduced by a CAF concentration of 4 μM ($P<0.001$ 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM) (fig.21, d and e).

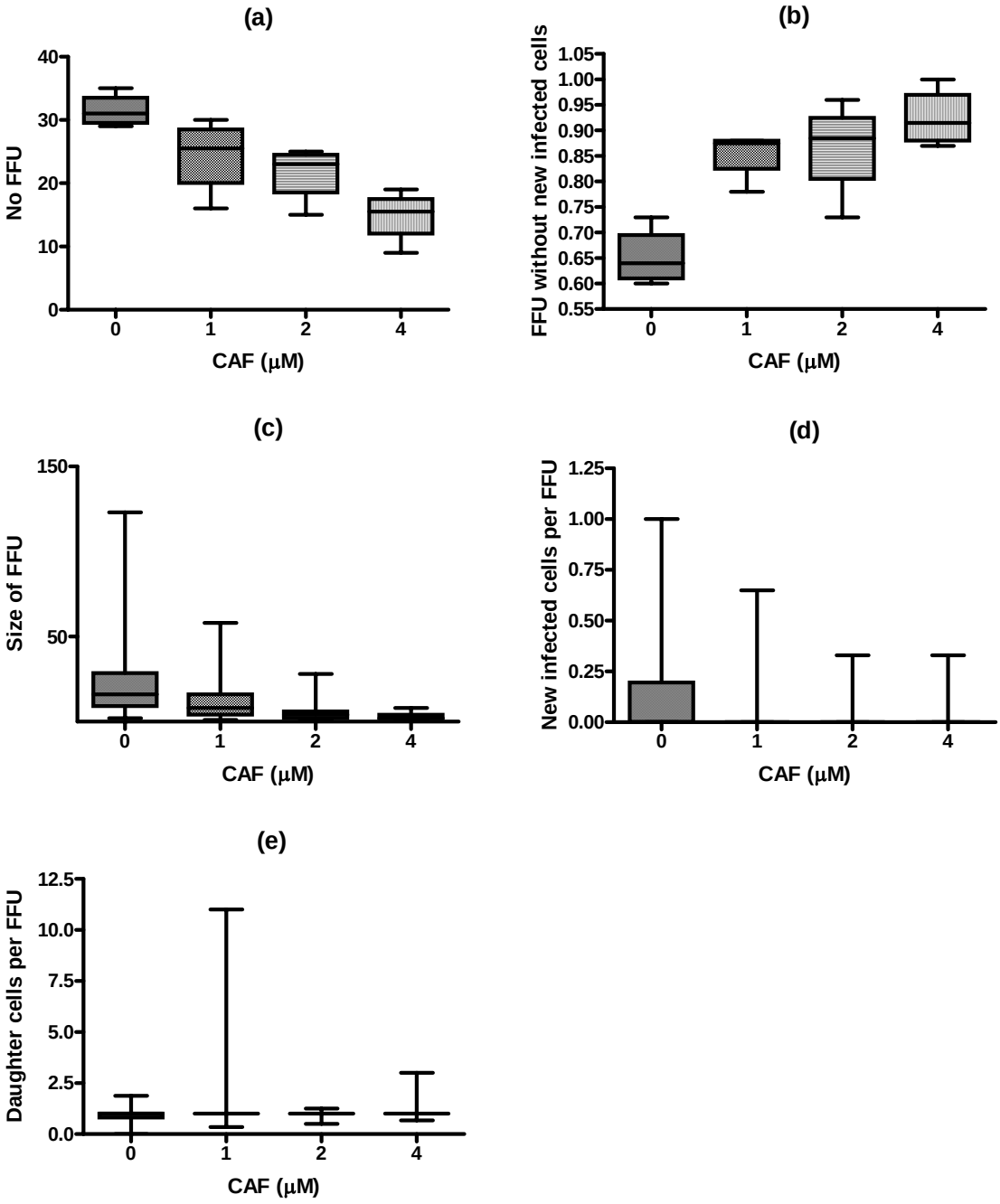


Fig. 21: Effect of CAF on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.01$) for 0 vs. 4 μM (b) The number of FFU without new infected cells is reported as a function of the concentration of CAF. A statistically significant difference ($P<0.01$) was detected for 0 vs. 1 and 0 vs. 2; $P< 0.001$ for 0 vs. 4 and 1 vs. 4 μM. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of CAF. A statistically significant difference was detected ($P<0.001$) for 0 vs. 1, 0 vs. 2, 0 vs. 4, 1 vs 4; $P<0.01$ for 1 vs. 2 μM. (d) The number of new infected cells per FFU is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. (e) The number of daughter cells per FFU is reported as a function of the concentration of CAF. A statistically significant difference was detected ($P< 0.05$) for 0 vs. 1 μM. See fig. 7 for key.

CAF was tested on BDV infected and non infected Vero cells at the concentrations of 1, 2, 4 μM. A decreased number of FFU, statistically significant ($P<0.05$) was detected at the concentration of of 0 vs. 1, 0 vs. 2, 0 vs. 4 μM (fig.22 a).

The number of FFU composed exclusively of daughter cells decreased with a statistically significant difference ($P<0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM (fig.22 b).

Also the size of the FFU decreased statistically significant ($P<0.001$) for concentration of CAF of 0 vs. 1, 0 vs. 2, 0 vs. 4 (fig.22 c).

The same significant decrease has been observed in the number of daughter cells per FFU ($P<0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM and in the number of new infected cells per FFU ($P<0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM (fig.22 d and e). No dose dependent differences between the tested concentrations of CAF was noted.

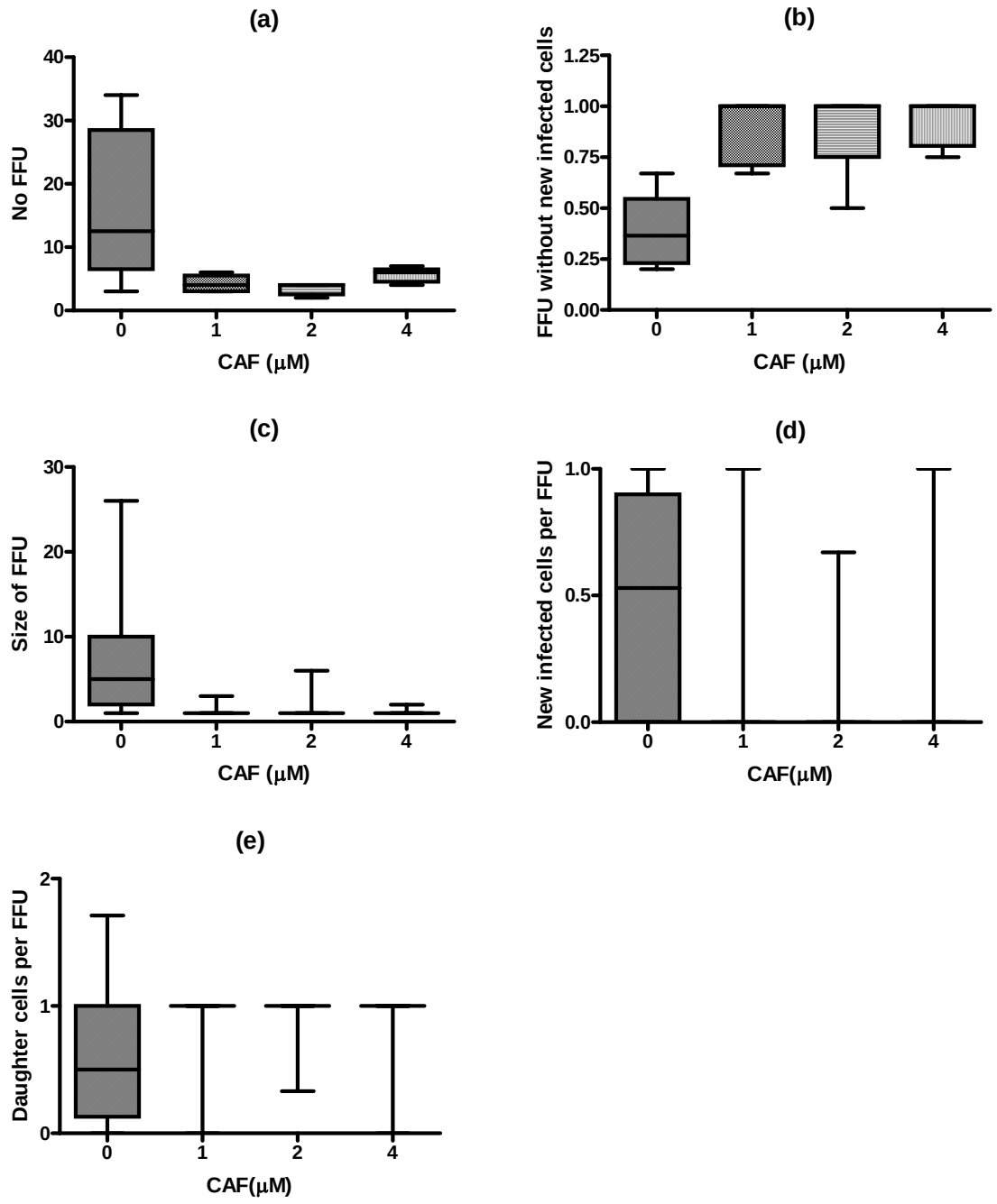


Fig. 22: Effect of CAF on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.05$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. (b) The number of FFU without new infected cells is reported as a function of the concentration of CAF. A statistically significant difference ($P<0.001$) was detected for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of CAF. Statistically significant difference was detected ($P<0.001$) for 0 vs. 1, 0 vs. 2, 0 vs. 4 μM. (d) The number of new infected cells per FFU is reported as a function of the concentration of CAF. A statistically significant difference was detected ($P<0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. (e) The number of daughter cells per FFU is reported as a function of the concentration of CAF. A statistically significant difference was detected ($P < 0.001$) for 0 vs. 1, 0 vs. 2 and 0 vs. 4 μM. See fig. 7 for key.

4.4.3 Amantadine

Amantadine was tested on BDV infected and non infected MDCK cells at the following concentrations: 0, 16, 32, 64 μM. None of the considered parameters showed any variations when MDCK cells were incubated with Amantadine (fig.23, a - e).

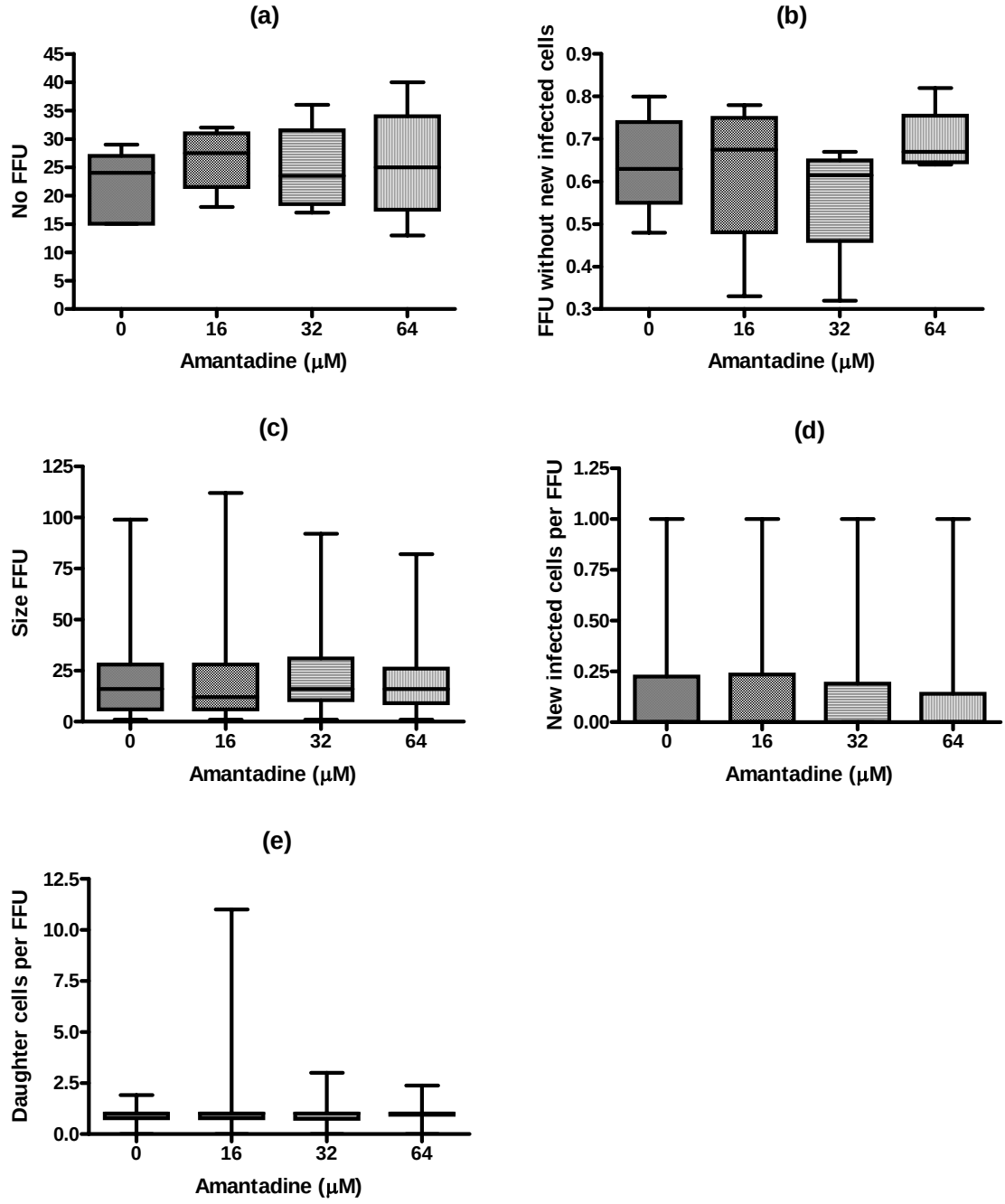


Fig. 23: Effect of Amantadine on BDV infected and non infected MDCK cells. (a) The number of FFU is reported as a function of the concentration of Amanatadine. No statistically significant difference was detected. (b) The number of FFU without new infected cells is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (d) The number of new infected cells per FFU is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (e) The number of daughter cells per FFU is reported in function of the concentration of Amantadine. No statistically significant difference was detected. See fig. 7 for key.

Amantadine was tested on BDV infected and non infected Vero cells at the following concentrations: 0, 16, 32, 64 μM.

The number of FFU remained unchanged (fig.24 a) as the number of FFU composed exclusively of daughter cells with the addition of Amantadine (fig. 24 b).

A decrease in the size of the FFU has been detected at the following concentrations: $P<0.01$ for 0 vs. 16 and $P<0.001$ for 0 vs. 64 μM (fig.24 c).

The number of daughter cells per FFU did not showed any change, while the number of new infected cells is significantly decreased: $P< 0.05$ for 0 vs. 16 and 0 vs. 64 μM, and $P<0.01$ for 0 vs. 32 μM (fig.24 d and e).

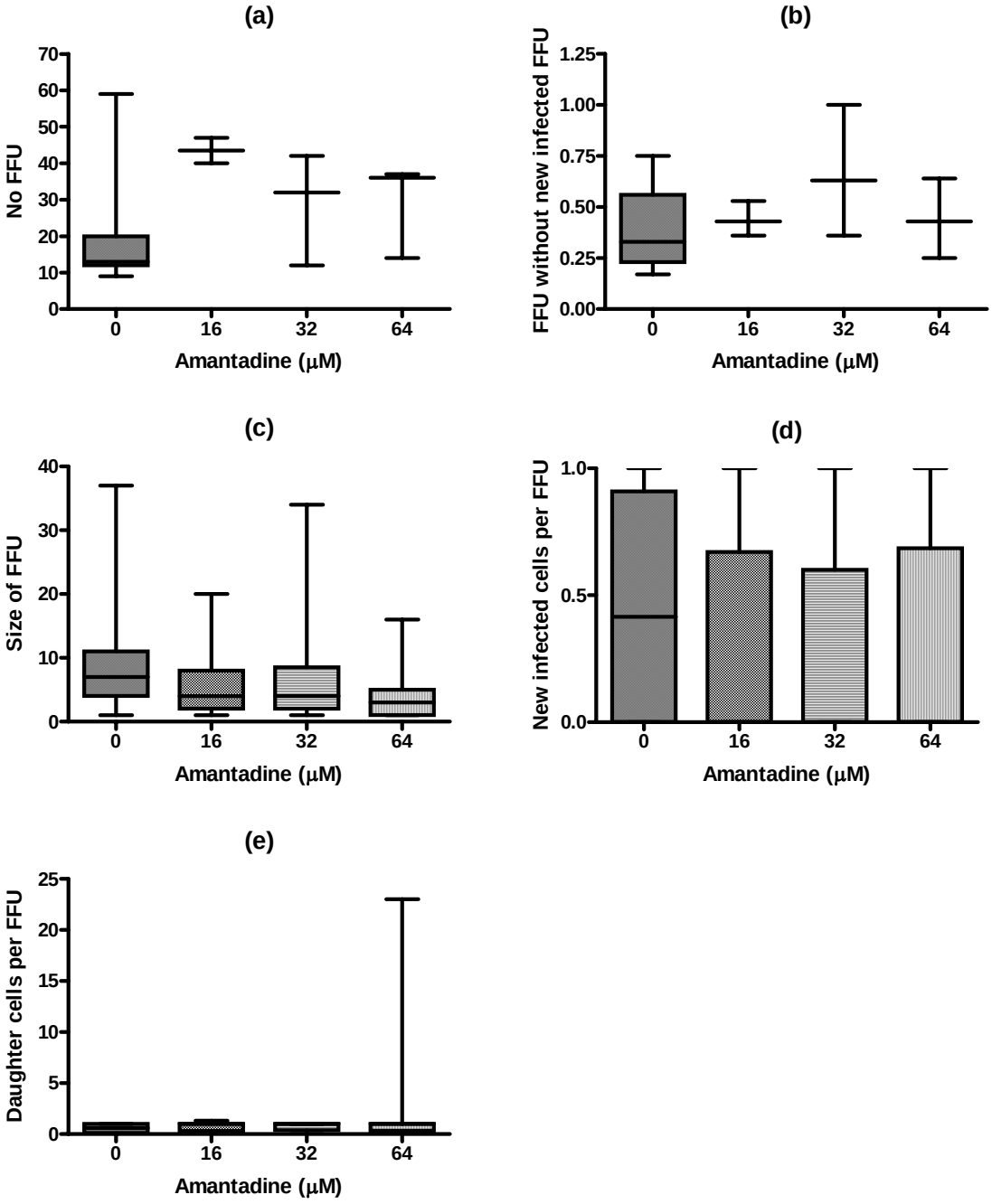


Fig. 24: Effect of Amantadine on BDV infected and non infected Vero cells. (a) The number of FFU is reported as a function of the concentration of Amanatadine. No statistically significant difference was detected. (b) The number of FFU without new infected cells is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. (c) The size of the FFU (expressed as the number of BDV infected cells per FFU) is reported as a function of the concentration of Amantadine. Statistically significant difference was detected for 0 vs.16; $P<0.001$ for 0 vs. 64 μM. (d) The number of new infected cells per FFU is reported as a function of the concentration of Amantadine. A statistically significant difference was detected ($P<0.05$) for 0 vs. 16 and 0 vs. 64; $P<0.01$ for 0 vs. 32 μM. (e) The number of daughter cells per FFU is reported as a function of the concentration of Amantadine. No statistically significant difference was detected. See fig. 7 for key.

5. Discussion

5.1 Antiviral activity of proteins

In this study both the native and modified albumin strongly impaired the cell growth. Chemically modified Lactoferrin, β-lactoglobulin and lysozyme proteins did also inhibit or markedly impair the cellular replication to such an extend that no cellular monolayer was obtained after three days of incubation, thus making an evaluation of a potential antiviral activity very difficult or even impossible.

In previous studies, the proteins which we tested had shown a marked antiviral activity against Human Herpes Simplex Virus type 1 [102], HIV and Human Cytomegalovirus [56], Adenovirus [4] and other viruses (see 4.2).
Chemical modification through reaction with 3-HP of these proteins yields compounds which strongly inhibited the multiplication of Herpes Simplex Virus-1.
However, since 3-HP-modified proteins failed to inhibit Bovine Parainfluenza Virus type 3 and Porcine Respiratory Coronavirus (all enveloped viruses as BDV) this indicates that an inhibition by unspecific damage of the virus envelope, caused by hydrophobic and electronegative interaction between the 3-HP-proteins and the envelope proteins, is unlikely. The native proteins and the chemical compound 3-HP alone did not show any antiviral activity against the virus investigated, indicating that the inhibition of the cytopathic effect of HSV-1 was a peculiar property of the modified proteins [103].

5.2 Ribavirin

Our investigations confirmed the antiviral activity against BDV of Ribavirin. Ribavirin proved to be active against BDV in persistently infected cells on both MDCK and Vero cells.
The size of the FFU (expressed as the number of BDV infected cells per FFU) was reduced only on MDCK cells. This can be interpreted as a different sensitivity to Ribavirin depending on the cell line. It was interesting to investigate if the antiviral effect was a consequence of a reduction in the cell replication and consequently of

the viral transmission or due to an inhibition of the virus spread from cell to cell independent from the cell cycle.
In order to assess, whether the infection was carried out mainly through inheritance of the virus in the cells progeny or through the passage of the virus from BDV infected to non infected cells, the cells were labelled with CMTPX and every single FFU was examined.
Our result showed that the FFU were composed mainly of daughter cells that transmit the infection through their replication, confirming that the infectivity of the BDV for new cells is very low.
Nevertheless, the proportion of infected cells that did not derive from the original persistent infected monolayer could be quantified, showing that in this case the size reduction of the FFU was attributable to a parallel reduced number of new infected cells. As a consequence, the number of FFU without newly infected cells was slightly increased.
Interestingly, the number of newly infected cells per FFU was the only parameter that statistically significantly decreased also in tests with Vero cells, but evidently not enough to determine a total variation of the progress of the infection.
It appears therefore likely that the antiviral effect of Ribavirin can be attributed to a reduction of the virus spread from cell to cell and that this is partially dependent on the cell line used.

5.3 CAF

In this study, the strong antiviral anti-BDV activity of CAF previously reported [7, 141] (see 2.9.2) could be confirmed.
After incubation of CAF with persistently infected cells, only MDCK cells, but not Vero cells, showed a significant reduction of the percentage of infected cells, suggesting that also in this case the sensitivity to CAF treatment is dependent on the cell line.
On other hand, both the number and the size of the FFU were significantly decreased in the presence of CAF during the incubation on both MDCK and Vero cell lines.

On both cell lines the FFU showed a tendency to be composed exclusively of daughter cells when incubated with CAF. This observation suggests that the virus spread form cells to cell can be limited or impaired by the presence of CAF.
Additionally, both the number of new infected cells and the number of daughter cells per FFU were reduced showing that CAF probably can have an effect on the virus spread as well as on the multiplication of the infected cells. Considering however that the monolayer is completed after the standard incubation time, it can be excluded that the cytostatic effect of CAF would significantly interefere with the cells growth.

5.4 Amantadine

In this study, we could find a statistically significant antiviral effect of Amantadine at the concentration of 64 μM only on BDV persistently infected MDCK cells. The same concentrations tested on Vero cells did not show any effect.
On the other hand, incubation with Amantadine caused a reduction in the number of new infected cells per FFU and a reduced average size of the FFU on Vero cells, but the same concentration failed to show any effect on MDCK cells.
These results firstly support the idea that different cell lines could have a different sensitivity to Amantadine, as previously presumed [16, 43](see 2.9.3).
Moreover, the statistically significant reduction of the infected cells per FFU was detectable only in the experiment performed with the CMTPX, and not in the assays where BDV infected cells were not labelled.
This suggests that CMTPX and Amantadine could have a synergic effect on Vero cells. However, this effect was not noticed on MDCK cells and needs to be further investigated.

5.5 Concluding remarks

The main results of our study can be summarized as follows:

- Confirmed antiviral effect of Ribavirin on BDV persistently infected MDCK cells and on the size of the FFU. This activity could be demonstrated to be exerted through an impairment of the virus spread from cell to cell;
- Antiviral effect of Ribavirin on BDV persistently infected Vero cells
- Confirmed anti-BDV effect of CAF on BDV persistently infected MDCK cells and on the number and size of FFU on both MDCK and Vero cell lines. This antiviral activity has been demonstrated to be exerted through an impairment of the virus spread from cell to cell as well as on an inhibition of the replication of infected cells, on both the cell lines;
- Antiviral effect of Amantadine on BDV persistently infected MDCK cells, but not on persistently infected Vero cells
- Antiviral effect of Amantadine on the size of FFU on Vero cells, due to a reduction of the virus spread from cell to cell. This effect was not observed on MDCK cells

Given the assumption that the cells growth is a biological, dynamic process and therefore hardly ever constant, even if the cultivation procedure is in itself standardized, every cell monolayer is somehow unique and different from all the others. The ideal cell monolayer is composed by definition of a single, uniform layer of cell that allows a good identification of the cells border and morphology. The numerical evaluation of the cell culture slides was limited by various factors: On each well of the slides there are inhomogenous areas, where the cell density is higher or lower, or where a certain degree of background reaction was present, a fact that made the counting procedure sometimes difficult.
Moreover, the multiple fluorescent granules characterizing the BDV infected cells are typically present in the nucleus. Nevertheless, because of the partial overlapping of the cells, sometimes the exact number of positive cells was difficult to assess.
Data issued from optical cell counts are generally prone to a certain degree of subjectivity, however this was sensibly reduced through the fact that the same person carried out all the experiments in a relatively short period of time.

CMTPX proved to be a useful marker to discriminate vertical and horizontal transmission of the virus in cell culture. A proportion of cells was labelled with CMTPX but did not appear BDV infected by immunofluorescence.
In fact, these cells could be either part of the original BDV persistently infected cell line, that did not contain the virus (in a BDV persistently infected monolayer the majority, but not all of the cells are positive at immunofluorescence), or that they had lost the virus during the replication process.

Another difficulty of this study was to establish a threshold of cytotoxicity when the cells were incubated with the compounds tested. As a rule, we excluded in the study those slides in which the monolayers showed diffuse changes in the cell morphology (shape and size) or in the cell density compared to the negative control. Optical control does not exclude variations in the kinetics of the cell growth. Confluence of the monolayers was always complete after 3 days of incubation, but might be reached earlier in non treated than in treated samples. Considering the fact that BDV requires a tight contact between the cells for a successful spread and that the infectivity of the virus is quite low [30], the question remains, if a delayed contact, due for example to a retardation of the cell growth, could affect the virus spread resulting in a impaired transmission of the infection from cell to cell.

In spite of these limitations, this study clearly demonstrated an inhibition of virus growth and spread in cell culture by various antiviral compounds, such as Ribavirin, CAF and Amantadine, as well as a differential sensitivity of the two cell lines used.

6. References

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7. Abbreviations

1B6TM	1-0-benzyl-6-0-trityl-α-D-mannopyranoside
3-HP	3-Hydroxypthalic anhydride
BD	Borna Disease
BDV	Borna Disease Virus
CAF	Cytosine β-D-arabinofuranoside
CFSA	5 (and 6) carboxyfluoroscein diacetate, succinimidyl ester
CNS	Central Nervous System
CSA	Cyclosporine A
CSF	Cerebrospinal fluid
CSFF	Cerebrospinal fluid filtration
FFU	Focus Forming Unit
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFN	Interferon
IIF	Indirect Immunofluorescence
MDCK	Madin Darby Canine Kidney cells
NNS RNA	Negative non-segmented Ribonucleic acid
PBS	Phosphate Buffer Solution
PO	phosphodiester oligonucleotide
RNP	viral ribonucleoparticles

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9. Curriculum Vitae

Name	Francesca Del Chicca
Geburtsdatum	20. Mai 1978
Geburtsort	Parma, Italien
Nationalität	Italien
Heimatort	IT-43100 Parma, v. XXII Luglio 2
1984-1989	Primarschule Parma
1989-1992	Sekundarschule Parma
1992-1997	Gymnasium Liceo Scientifico 'G. Marconi' Parma
1997	Abitur (Maturità scientifica)
1997-2002	Studium der Veterinärmedizin an der Universität Parma (Università degli Studi di Parma)
2002	Assistentin in Kleintierpraxis 'Miller' in Cavriago RE, IT
2002-2003	Austauschstudentin im Rahmen des Erasmus-Programmes an der Vetsuisse-Fakultät Universität Zürich
2003	Staatsexamen, Italien
2003	Assistentin in Gross- und Kleintierpraxis Ambleside Veterinary Clinic in Worcester, UK
2004-2006	Doktorandin bei Prof. Dr. F. Ehrensperger am Institut für Veterinärpathologie, Abteilung Immunopathologie, Vetsuisse-Fakultät der Universität Zürich
Seit Juli 2006	Resident am Institut für Radiologie und bildgebende Diagnostik, Vetsuisse-Fakultät der Universität Zürich

10. Annex

Table 1. Percentage of BDV infected cells per field incubated with different concentrations of Ribavirin, CAF and Amantadine

		MDCK cells		Vero cells	
	μM	Percentage of infected cells per field	Statistical significance	Percentage of infected cells per field	Statistical significance
Ribavirin	0	94.30±6.20	P<0.01 for 0 vs. 8	95.00±8.87	P<0.05 for 0 vs 8
	1	89.10±5.74		91.70±4.64	
	4	81.00±10.98		88.50±7.00	
	8	74.00±10.51		83.60±12.91	
CAF	0	94.30±6.20	P<0.05 for 0 vs. 1 P<0.01 for 0 vs. 4 0 vs. 8	98.60±1.90	-
	1	81.70±13.78		97.90±2.47	
	4	77.90±9.36		98.10±0.88	
	8	72.30±5.80		97.70±2.36	
Amantadine	0	97.00±2.12	P<0.01 for 0 vs. 64	94.90±4.18	-
	16	87.40±7.50		89.80±8.44	
	32	88.40±4.61		88.60±5.38	
	64	88.90±9.93		90.90±8.28	

Table 1: The reported data are mean, standard deviation and statistical significance (data are processed using the statistical application program 'Prisma', in particular through the Bonferroni's multiple comparison test). Results with a P<0.05 were assumed to be significant. The different statistical significances as well, are reported.

Table 2. Number and size* of FFU when MDCK and Vero cells are incubated with different concentrations of Ribavirin, CAF and Amantadine.

		MDCK cells				Vero cells			
	μM	No FFU	Statistical significance	Size* FFU	Statistical significance	No FFU	Statistical significance	Size* FFU	Statistical significance
Ribavirin	0	37.00±16.79	-	27.78±20.97	P<0.001 for 0 vs. 4, 0 vs. 8, 1 vs. 4, 1 vs. 8	29.00±2.94	-	6.91±5.54	-
	1	37.00±12.03		26.32 (0.00, 52.92)		23.25±7.27		5.70±4.70	
	4	34.00±18.74		14.84±10.76		26.00±3.92		5.34±4.29	
	8	17.50±9.61		9.71±8.57		22.50±2.38		5.24±3.79	
CAF	0	43.25±11.15	P<0.01 for 0 vs. 4 0 vs. 8	28.37±21.81	P<0.001 for 0 vs. 1, 0 vs. 4, 0 vs. 8, 1 vs. 4, 1 vs. 8	20.50±2.89	P< 0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4	6.11±5.56	P< 0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4
	1	28.75±9.91		17.98±16.80		5.75±4.11		1.09±0.29	
	2					4.25±3.20		1.29±0.69	
	4	13.25±10.18		4.81±3.26		4.75±1.26		1.00±0.00	
	8	9.50±5.97		2.56±1.61					
Amantadine	0	48.93±11.98	-	23.37±18.97	-	56.25±10.84	-	6.36 (0.00,12.77)	-
	16	44.44±17.36		23.38±19.74		51.75±1.70		7.55 (0.00,15.35)	
	32	35.00±18.06		27.05±22.81		60.00±10.03		6.36 (0.00,12.97)	
	64	50.38±14.70		19.15±17.40		53.75±7.14		6.49±5.74	

Table 2: Data are reported as mean and standard deviation. Data are processed using the statistical application program 'Prisma', in particular through the Bonferroni's multiple comparison test. Results with a P<0.05 were assumed to be significant. The different statistical significances as well, are reported.
*: size of FFU is expressed as the number of BDV infected cells per FFU.

Table 3. Total number of FFU, number of FFU without new infected cells, size* of the FFU, number of new infected cells per FFU and number of daughter cells per FFU when MDCK cells are incubated with the different concentrations of Ribavirin, CAF and Amantadine.

		MDCK									
	μM	No of FFU	Statistical Significance	No of FFU without new infected cells	Statistical Significance	Size* of FFU	Statistical Significance	New infected cells per FFU	Statistical Significance	Daughter cells per FFU	Statistical Significance
Ribavirin	0	23.23±13.69	-	0.66±0.17	P<0.05 for 0 vs. 8	27.79±21.34	P<0.01 for 0 vs. 4 P< 0.001 for 0 vs. 8 1 vs. 8 P<0.05 for 1 vs. 4	0.07 (0.00, 0.26)	P<0.05 for 0 vs. 8 1 vs. 4 P<0.01 for 1 vs. 8	1.05 (0.00, 2.16)	-
	1	21.36±13.58		0.69±0.14		27±23.21		0.09 (0.00, 0.32)		0.99±0.83	
	4	18.43±9.89		0.74±0.19		22.07±19.50		0.03 (0.00, 0.16)		1.05±0.59	
	8	13.50±10.39		0.86±0.14		16.74±13.10		0.01 (0.00, 0.11)		1.13±0.92	
CAF	0	31.50±2.65	P<0.01 for 0 vs. 4	0.65±0.06	P<0.01 for 0 vs. 1 0 vs. 2 P< 0.001 for 0 vs. 4 1 vs. 4	22.58±19.71	P<0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4, 1 vs. 4 P<0.01 for 1 vs. 2	0.15 (0.00, 0.42)	P<0.001 for 0 vs. 1, 0 vs. 2 0 vs. 4	0.86±0.29	P<0.05 for 0 vs. 1
	1	24.25±6.02		0.85±0.05		11.65±10.31		0.04 (0.00, 0.16)		1.09±1.05	
	2	21.50±4.51		0.87±0.10		4.85±3.98		0.02 (0.00, 0.09)		0.98±0.10	
	4	14.75±4.19		0.93±0.06		2.93±1.72		0.01 (0.00, 0.07)		1.04±0.30	
Amantadine	0	22.43±5.48	-	0.63±0.11	-	19.30±16.57	-	0.15 (0.00, 0.42)	-	0.86±0.31	-
	16	26.25±6.24		0.62±0.20		19.59 (0.00,40.33)		0.18 (0.00, 0.52)		0.97 (0.00, 2.10)	-
	32	25.00±8.45		0.56±0.16		22.74±18.71		0.15 (0.00, 0.43)		0.85±0.39	
	64	25.75±11.32		0.70±0.08		19.24±13.90		0.16 (0.00, 0.47)		0.89±0.36	

Table 3: Data are reported as mean and standard deviation. Data are processed using the statistical application program 'Prisma', in particular through the Bonferroni's multiple comparison test. Results with a P<0.05 were assumed to be significant. The different statistical significances as well, are reported. *: size of FFU is expressed as the number of BDV infected cells per FFU.

Table 4. Total number of FFU, number of FFU without new infected cells, size* of the FFU, number of new infected cells per FFU and number of daughter cells per FFU when Vero cells are incubated with the different concentrations of Ribavirin, CAF and Amantadine.

	μM	Vero cell									
		No of FFU	Statistical Significance	No of FFU without new infected cells	Statistical Significance	Size* of FFU	Statistical Significance	New infected cells per FFU	Statistical Significance	Daughter cells per FFU	Statistical Significance
Ribavirin	0	12.75±12.72	-	0.30±0.24	-	7.40 (0.00, 15.78)	-	0.50±0.41	P<0.05 for 0 vs. 1	0.47±0.41	-
	1	8.91 (0.00, 19.22)		0.46±0.24		8.48 (0.00, 17.87)		0.37(0.00, 0.77)		0.56±0.42	
	4	10.83±10.32		0.37±0.21		7.95±7.04		0.44±0.41		0.47±0.41	
	8	9.75±9.96		0.48±0.28		6.80 (0.00,13.96)		0.44±0.41		0.65 (0.00, 2.21)	
CAF	0	15.83±10.98	P<0.05 for 0 vs. 1, 0 vs. 2, 0 vs. 4	0.38±0.16	P<0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4	7.48±6.48	P<0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4	0.49±0.42	P<0.001 for 0 vs.1, 0 vs. 2, 0 vs. 4	0.54±0.44	P<0.001 for 0 vs. 1, 0 vs. 2, 0 vs. 4
	1	4.17±1.17		0.90±0.15		1.24±0.52		0.06 (0.00, 0.28)		0.94±0.22	
	2	3.40±0.90		0.92±0.20		1.37±1.17		0.04 (0.00, 0.19)		0.96±0.15	
	4	5.67±1.03		0.94±0.11		1.06±0.23		0.09 (0.00, 0.38)		0.97±0.17	
Amantadine	0	20.00±15.68	-	0.39±0.20	-	8.30±6.90	P<0.01 for 0 vs. 16 P<0.001 for 0 vs. 64	0.45±0.42	P<0.05 for 0 vs. 16, 0 vs. 64 P<0.01 for 0 vs. 32	0.56±0.42	-
	16	43.50±4.95		0.44±0.09		5.47±4.71		0.31 (0.00, 0.69)		0.67±0.39	
	32	28.67±15.28		0.66±0.32		7.04 (0.00,14.24)		0.28 (0.00, 0.64)		0.70±0.38	
	64	29.00±13.00		0.44±0.20		3.75±3.09		0.31 (0.00, 0.70)		0.90 (0.00, 3.02)	

Table 4: Data are reported as mean and standard deviation. Data are processed using the statistical application program 'Prisma', in particular through the Bonferroni's multiple comparison test. Results with a P<0.05 were assumed to be significant. The different statistical significances as well, are reported. *: size of FFU is expressed as the number of BDV infected cells per FFU.